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Krishnappa, Laxmi

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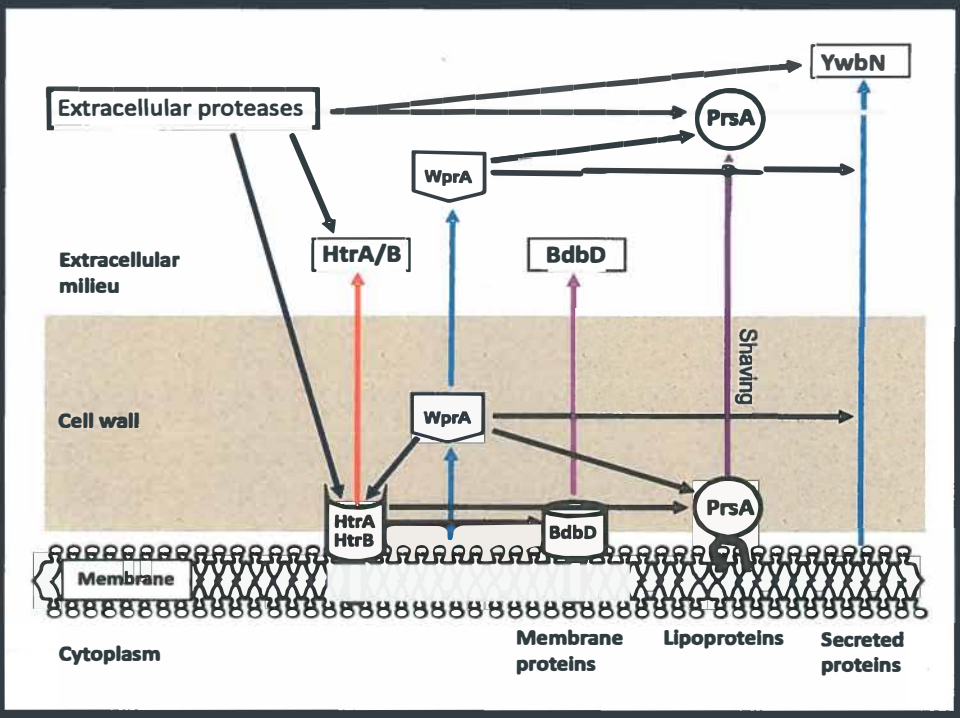

Laxmi Krishnappa

Proteolysis of extracytoplasmic proteins in *Bacillus subtilis*

Stellingen

Behorende bij het proefschrift

Proteolysis of extracytoplasmic proteins in *Bacillus subtilis*

1. Exoproteome analyses show that many secreted proteins, lipoproteins and membrane proteins of *B. subtilis* are potential substrates of extracytoplasmic proteases (this thesis).
2. The quality control proteases HtrA and HtrB are crucial for maintaining the integrity of the *B. subtilis* cell even under non-stress conditions (this thesis).
3. The observation that multiple extracellular proteases degrade the *B. subtilis* Tat substrate YwbN can be related to the fact that it is active in a complex with the membrane protein YwbL and the lipoprotein YwbM (Thesis Carmine G. Monteferrante and this thesis).
4. The protein quality control factors PrsA, HtrA and HtrB of *B. subtilis* are substrates of multiple extracytoplasmic proteases (this thesis).
5. Improved protein secretion by multiple protease mutant *B. subtilis* strains may relate to reduced proteolysis and elevated chaperone levels (this thesis).
6. Super-secreting *B. subtilis* strains lack multiple extracytoplasmic proteases (this thesis).
7. The proteolytic system of *B. subtilis* is complex and full of surprises.
8. Bacilli have a clean shave every day.
9. Protease inhibitors may have adverse effects on the virulence of pathogenic Gram-positive bacteria.
10. " Speech is silver, Silence is golden, Sleep is platinum"- Laxmi Krishnappa
11. " There is nothing lost or wasted in this life"- Bhagvad Gita

Laxmi Krishnappa

Bangalore, 10 January 2013

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Schematic representation of extracytoplasmic proteases and their potential targets in *Bacillus subtilis*. Cover designed by Laxmi Krishnappa and Jan Maarten van Dijl.

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Proteolysis of extracytoplasmic proteins in *Bacillus subtilis*

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This thesis is dedicated to my teachers

Contents

Chapter 1

Introduction and scope of the thesis

Chapter 1: Introduction and scope of the thesis

Bacillus subtilis is a Gram-positive bacterium that inhabits the upper layers of the soil and the plant rhizosphere [1]. In these ecological niches, *B. subtilis* is frequently exposed to harsh environmental situations, such as draught, extreme temperature changes, fluctuating osmolarity and nutrient limitation. *B. subtilis* employs several adaptive strategies to cope with harsh environmental challenges, or to avoid them altogether. For example, synthesis of a flagellum enables *B. subtilis* to migrate towards favorable environments [2, 3, 4]. Antibiotic production and degradative enzyme secretion by *B. subtilis* ensure a competitive advantage in relation to other organisms in the soil [5, 6, 7]. Furthermore, *B. subtilis* can easily become competent to take up foreign genetic material as a nutrient or to increase its genetic diversity, which can be helpful for survival of the species [8, 9]. The uptake of K^+, increased synthesis of proline and uptake of glycine betaine ensures protection during changes in environmental osmolarity [10-14]. If moderate adaptations are insufficient for cell survival, particular cells will produce highly resistant endospores that ensure the viability of *B. subtilis* in a dormant state until favorable situations are encountered [15, 16, 17].

Today, *B. subtilis* is the best-studied Gram-positive rod-shaped bacterium in the scientific world due to the early availability of its complete genome sequence [18, 19]. Therefore, *B. subtilis* is an ideal model organism for studies on chromosomal replication, gene regulation, metabolism, protein secretion and bacterial cell differentiation (e.g. motility, competence development and sporulation). In addition, the applied methods for such studies and the outcome are useful for research on closely related Gram-positive pathogens, such as *Staphylococcus aureus*. Systems biology approaches in the areas of transcriptomics and metabolomics have revealed the highly versatile abilities of *B. subtilis* [20, 21].

The availability of the genome sequence not only enhanced the understanding of basic scientific processes, but has also supported *B. subtilis* to emerge as the model organism for the study of heterologous protein secretion. The extraordinary ability of *Bacillus* species to secrete large quantities (20-25g/L) of protein into the culture medium makes it ideal for exploitation in the biotechnological industry for the production of commercially important products, like enzymes used in washing powders and leather industry. In addition, *B. subtilis* has become the workhorse for the production of vitamins, like riboflavin and biotin. Since *B. subtilis* is non-pathogenic it is Generally Regarded As Safe (GRAS). Therefore, *B. subtilis* is currently explored as an attractive (endo-) toxin-free cell factory for promising biopharmaceuticals, like cytokines or scyllo-inositol for the treatment of Alzheimer's disease and many more [22-25].

The secretome of *Bacillus subtilis*: biological and commercial relevance

Signal peptides and signal peptidases

Notably, many applications of *B. subtilis* in the field of biotechnology are related to the high-level secretion of proteins. This has focused major research interests on the secretome, which includes both the protein secretion machinery and the secreted proteins [26]. *B. subtilis* secretes proteins for a wide range of purposes, including nutrient acquisition, competition with other organisms and adaptation to changes in the environment. The secreted proteins of *B. subtilis* usually contain N-terminal stretches of amino acid sequences called signal peptides that are important for export from the cytoplasm. These signal peptides are composed of three characteristic domains generally known as the N-, H- and C-regions [27-30]. Arginine or lysine residues in the N–region are known to interact with the translocation

machinery and the negatively charged phospholipids in the cell membrane [31, 32]. The H-region comprises hydrophobic residues. It adopts an alpha-helical conformation in the membrane, which is necessary for the initiation of protein translocation across the membrane [33]. The C-region contains the recognition and cleavage sites for signal peptidase (SPase). These SPases cleave off the signal peptide to release the mature protein from the membrane during or shortly after translocation via a dedicated secretory apparatus. The cleaved signal peptides are then targeted for degradation by the 'site-2' membrane protease RasP, and possibly other membrane-associated proteases [34].

Secreted proteins can contain one of the five different types of signal peptides identified in *B. subtilis*. These are i) twin-arginine (RR/KR) signal peptides, ii) secretory (Sec-type) signal peptides, iii) Lipoprotein signal peptides, iv) pseudopilin-like signal peptides, and v) bacteriocin and pheromone signal peptides [26]. The latter signal peptides are mostly referred to as leader peptides or leader sequences (Figure 1). Proteins with a twin-arginine signal peptide are translocated across the membrane via the Tat translocation machinery, whereas proteins with a Sec-type signal peptide are translocated via the Sec machinery. The so-called type I SPases commonly target twin-arginine and Sec-type signal peptides. In fact, these signal peptides can be cleaved by one of the five known SPases of *B. subtilis* (i.e. SipS-W) [35, 36]. Pre-lipoproteins are generally exported via the Sec pathway and the respective signal peptides are cleaved by type II SPases (also referred to as lipoprotein SPases or LspA), but only after the signal peptide has been lipid-modified [37, 38]. The pseudopilins ComGC, ComGD, ComGE and ComGG are translocated via the Com pathway and retained in the cell membrane. These pseudopilins possess pseudopilin-like signal peptides that are cleaved by the pseudopilin-specific SPase ComC [26, 39, 40]. Finally, the

leader peptides of pheromones, such as ComX, and the lantibiotic sublancin are cleaved and translocated by the respective ABC transporters that facilitate their secretion [41, 42].

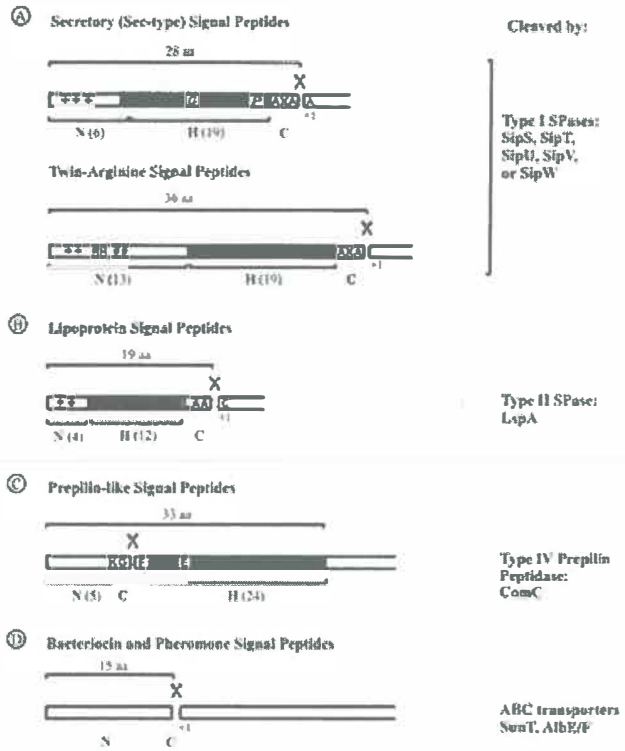

Figure 1. The signal peptides of *B. subtilis* and other organisms consist of 3 domains namely the N-, H- and C- domains. Generally, the N-domain contains positively charged residues for binding to the membrane and to protein translocation machinery components. The hydrophobic H-domain is needed for membrane insertion of the signal peptides. In some signal peptides, the H-domain contains the helix-breaking residues proline/glycine. The C-domain consists of sequences for recognition and cleavage by different signal peptidases. **A,** The Sec-type signal peptide is the commonly found signal peptide associated with the majority of secretory proteins in *B. subtilis*. The N-terminus contains up to 11 positively charged residues, usually lysine (K) or arginine (R). The N-terminus of the Tat-type signal

peptides includes a twin-arginine (RR-) motif. The C-termini of both the Sec- and Tat-type signal peptides contain the AXA consensus recognition sequence for type I signal peptidases, depicted as scissors. **B,** The signal peptides of lipoproteins are smaller compared to Sec- and Tat-type signal peptides. The unique 'lipobox' is present in the C-domain. It contains the indispensable cysteine residue that is required for lipid modification. Type II signal peptidases target the lipoprotein-type signal peptide at the lipobox, subsequently cleaving N-terminally of the lipid-modified cysteine residue. **C,** Pseudopilin signal peptides have a C-domain that is positioned between the N- and C- domains. This relates to the fact that pseudopilin signal peptidases like ComC of *B. subtilis* act at the cytoplasmic side of the membrane. **D,** Bacterocin and pheromone signal peptides consist of N- and C-domains, and lack a hydrophobic H-hydrophobic domain [43]. This Figure was adapted from Tjalsma et al 2000 [26].

Protein transport pathways

As indicated above, the various signal peptides of secreted proteins are specifically recognized by dedicated translocation machineries. The most relevant and best-studied translocation pathways for secretory proteins in *B. subtilis* are the general secretory (Sec) pathway and the twin-arginine translocation pathway (Tat) [26].

The Sec pathway: The majority of the secreted proteins are translocated from the cytoplasm to the exterior of *B. subtilis* via the highly conserved Sec pathway (Figure 2). During ribosomal synthesis the proteins secreted via Sec are synthesized with N-terminal signal peptides. The precursor proteins are maintained in a translocation-competent state by cytoplasmic chaperones like SRP and FtsY, enabling the translocation across the membrane through the Sec apparatus [43, 44, 45].

The Sec apparatus consists of SecA, the translocation motor, and the integral membrane proteins SecY, SecE, SecG and SecDF. The energy for translocation of proteins is derived from ATP binding by SecA and the proton-motive force (PMF). The ATPase activity of SecA is triggered by the interaction of the translocation-competent protein with SecA, the SecYEG translocation channel and the phospholipids of the cell membrane [46]. SecA then drives the protein through the SecYEG channel through cycles of ATP binding and hydrolysis [47]. The exact function of SecDF of *B. subtilis,* a 'molecular Siamese twin protein' is as yet unknown, but it is indeed required for efficient export, especially under conditions of secretory protein overproduction [48, 49].

Based on studies in *Escherichia coli* and *B. subtilis*, it seems likely that the SpoIIIJ and YqjG proteins can operate in concert with the Sec machinery in protein translocation. However, this seems to apply mainly to the translocation of membrane proteins. In addition, certain other membrane proteins may be inserted into the membrane in a non-Sec-dependent manner with the help of SpoIIIJ/YqjG [50-52].

The N-terminal signal peptides of precursor proteins are cleaved by SPases during or shortly after translocation via the Sec apparatus. Only after translocation through the Sec apparatus has been completed are the secreted proteins folded into a biologically active conformation by chaperones, such as PrsA [53] or the thiol-disulfide oxidoreductases BdbB, BdbC and BdbD [54, 55]. Other factors that are important for post translocational protein folding, are, propeptides and metal ions [56]. For example, the serine proteases of *B. subtilis* are guided by their propeptides to achieve their biologically active conformation. Propeptides also shield the serine proteases against proteolytic activities. Metal ions such as Ca^{2+}, Fe^{3+} and Mg^{2+} affect the folding and stability of many secreted proteins in *B. subtilis* [56-59]. Specifically, the requirement of Ca^{2+} for the stability of *Bacillus* subtilisin-like serine protease

has been demonstrated *in vitro* [60, 61]. Furthermore, the post-translocational folding and secretion of the model protein levansucrase was shown to require both Ca^{2+} and Fe^{3+} [62-64]. Lastly, the folded proteins can then fulfill their biological function at the membrane-cell wall interface, in the cell wall or in the external milieu. Whether the protein remains attached to the membrane will depend on the presence of a retention signal, such as a diacyl-glyceryl modification in the case of lipoproteins. Cell wall attachment will mainly depend on the presence of cell wall-binding domains, the covalent attachment to peptidoglycan or overall charge properties of the translocated protein. If a translocated protein lacks retention signals for the membrane or cell wall, it is usually effectively secreted into the extracellular milieu [65].The signal peptides and the Sec machinery of *B. subtilis* can be easily manipulated for exploitation in the biotechnology industry. Signal peptides of various proteins of *B. subtilis* could be potential candidates for the secretion of heterologous proteins. In this regard all natural signal peptides of *B. subtilis* were screened with the final conclusion that not every *B. subtilis* signal peptide is suited for the efficient secretion of a particular heterologous protein. Instead, there is a need to optimize the selection of the best-suited signal peptide for each particular heterologous protein [66].

The genetic engineering of the Sec machinery was shown to increase the secretion of some heterologous proteins. For example, the yield of human interferon-α2b was 2.2-fold higher in a strain with a deletion in the C-terminal linker (CTL) domain of SecA than the parental strain. Thus, the interactions between the engineered SecA and preproteins were apparently optimized for the secretion of hINF-α2b [67].

Recently, the *E. coli* SecB was coexpressed in a *B. subtilis* strain with a modified SecA protein. Specifically, the 32 C-terminal residues of SecA of *B. subtilis* were replaced by the corresponding amino acids of the *E. coli* SecA to optimize the SecA-SecB interaction. This engineering of the protein

secretion machinery resulted in the increased secretion of a mutant maltose-binding protein (MalE11) and the alkaline phosphatase PhoA of *E. coli*, which were used as model heterologous proteins [68]. It should be noted here that also early studies by Collier provided evidence that the heterologous expression of *E. coli* SecB in *B. subtilis* could be beneficial for the secretion of particular proteins [69].

The Tat pathway: The twin-arginine translocation pathway (Tat) is conserved in prokaryotes, chloroplasts of green plants and also in some archaea like *Haloferax volcanii* and *Haloarcula hispanica* [70-77]. Substrates with a highly conserved twin-arginine motif (RR-motif) in the N-terminal domain of the signal peptide are selectively chosen to be translocated via the Tat pathway [78-81]. Potential RR-motifs have been predicted by *in silico* analyses in the signal peptides of 69 *B. subtilis* proteins [80]. Proteomics and biochemical analyses have so far detected only PhoD, YwbN and YkuE as major substrates of the *B. subtilis* Tat system [82-84]. The proteins destined for translocation via the Tat pathway are usually folded prior to translocation. Thus, globular proteins and folded proteins with bound cofactors can be translocated via the Tat pathway across the cell membrane in a process that is driven by the proton-motive force. These unique characteristics distinguish the Tat pathway from the Sec pathway [85-87]. Notably, with the exception of streptomycetes, the Gram-positive bacteria have a Tat machinery that differs from the Tat machinery of Gram-negative bacteria with respect to the subunit composition. While Gram-negative bacteria have a three-component TatABC machinery, most Gram-positive bacteria have a two-component 'minimal' TatAC machinery[88-74] (Figure 2).

The TatA, TatB and TatC subunits are indispensable for the activity of the TatABC complex in *E. coli*. Studies in *E. coli* have also shown that the RR-motif is recognized by a TatBC sub-complex, and that this sub-complex

merges with an oligomeric TatA sub-complex to form the active TatABC translocation machinery [89-91]. The TatA sub-complex is believed to be responsible for the formation of a translocation pore in accordance with the size of the substrate. In *E. coli*, the TatA paralog TatE can substitute for TatA in the translocation of certain Tat substrates [91, 92]. The minimal TatAC type machinery of Gram-positive bacteria probably works in a similar manner but, in this case, the TatB subunit in the signal peptide receptor complex is replaced by a TatA subunit [88, 90, 91, 93, 94].

In contrast to *E coli*, *B. subtilis* contains two minimal TatAC translocases namely TatAyCy and TatAdCd that can function independently of each other. The *tatAy-tatCy* and *tatAd-tatCd* genes encoding the minimal translocases are situated as operons at different genomic locations [80, 88, 95, 96]. Recently, the third TatA subunit of *B. subtilis*, TatAc, was shown to form active complexes with both TatCd and TatCy [97].

PhoD, a protein with phosphodiestrase and alkaline phosphatase activity, is the only known substrate of the TatAdCd translocase. The *phoD* gene is located upstream of the *tatAd-tatCd* operon and it is only expressed during phosphate-limiting conditions [82, 88, 96, 98]. In contrast, the TatAyCy translocase is expressed constitutively. To date, the haem-containing Dyp-type peroxidase YwbN and the metallo-phosphoesterase YkuE are its only known substrates [96, 83]. During situations of hyper-production of particular proteins or when cells are grown at high salt concentrations, the Tat pathway seems to play a specific role in the secretion of proteins. For example, LipA is mostly a Sec substrate, but in a LipA hyper-producing strain, LipA can be secreted also via the Tat translocation pathway [80, 99]. Conversely, the Tat-dependent YkuE protein is secreted Tat-independently upon overproduction [83]. Furthermore, the YwbN protein is partially Tat-independently secreted when *B. subtilis* is grown in LB broth containing a high concentration of NaCl (6%), whereas it is strictly TatAyCy-dependently secreted under

standard laboratory conditions. Surprisingly, at high salinity growth conditions, TatAd is also involved in YwbN secretion in addition to the minimal TatAyCy translocase. In a medium without NaCl severe lysis was observed for *tatAyCy* or *ywbN* mutant cells, showing that under these conditions the TatAyCy translocase and its substrate YwbN are very important for cell growth and viability. This observation was shown to relate to an important role of Tat-dependent YwbN secretion in iron acquisition [100, 101].

The ability of the Tat machinery to secrete folded proteins makes it an attractive system for use in the biotechnology industry. Unfortunately however, the use of Tat signal peptides has, in many cases, not been very successful to achieve increased levels of secreted heterologous proteins. On the other hand, it has been shown that Sec-type proteins such as subtilisin can be re-routed into the Tat pathway [102]. Furthermore, a heterologous methyl parathion hydrolase (MPH) with the signal peptide of *E. coli* TorA was successfully translocated via the Tat machinery in *B. subtilis* [103]. Also, co-expression of TatAdCd or TatAyCy with hINF-α containing the LipA signal peptide in a Dpr8 strain (a *B. subtilis* strain deficient in 8 extracellular proteases) resulted in elevated levels of secreted hINF-α [23].

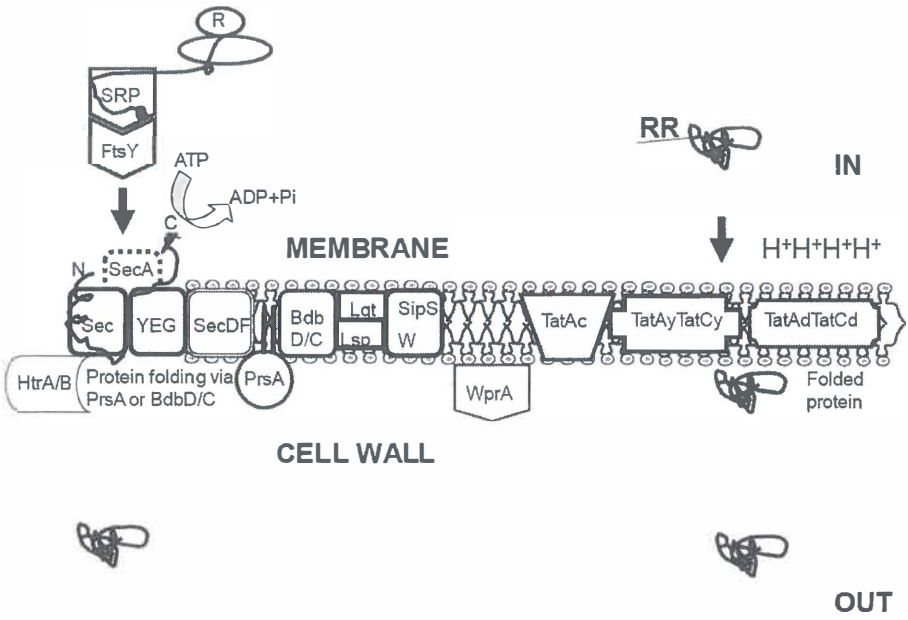

Figure 2. The Sec and Tat protein secretion pathways of *B. subtilis*. The cartoon depicts the components involved in Sec- and Tat-dependent export of proteins from the cytoplasm (IN) to the membrane, cell wall and extracellular milieu (OUT) of the bacterium. A nascent precursor protein is schematically presented as emerging from the ribosome and being bound by the SRP-FtsY complex for targeting to the membrane. Another precursor protein is schematically presented as being translocated via the SecYEG channel in an unfolded state. A folded precursor protein is schematically presented as being targeted to the Tat translocase. For further information on the roles of depicted pathway components, please see the main text. R, ribosome. RR, twin-arginine signal peptide.

Lipoprotein biogenesis in *Bacillus subtilis*

Lipoproteins in *B. subtilis* function in a wide range of processes, such as substrate-binding for transport via ABC transporters [104, 105], the maintenance of cell envelope homeostasis [106], protein folding and localisation [53, 107, 108], redox reactions [109-111] and sporulation [112]. In addition to this, closely related

Gram-positive pathogens, such as *S. aureus*, express lipoproteins that are known to induce invasion and phagocytosis and have an impact on intracellular survival in the host [113].

After translocation via the Sec pathway the prolipoporteins are lipid-modified by Lgt, a prolipoprotein diacyl-glyceryl transferase. This enzyme attaches the diacylglyceryl lipid anchor to the invariable cystein residue in the lipobox of prolipoproteins via a thioether linkage (Figure 1). Lipid modification is followed by the cleavage of the signal peptide at the conserved LspA cleavage site within the lipobox. After the cleavage by LspA, the lipoproteins are retained in the cell membrane via their N-terminal lipid moiety [114, 115].

Remarkably, lipoproteins are also identified in the extracellular proteome of *B. subtilis*. This initially unexpected, but widely conserved localization of lipoproteins is in most cases due to the loss of their lipid-modified N-terminal cysteine residues, which seems to relate to a unique "lipoprotein release" motif that was identified in some lipoproteins [108, 115].

PrsA is an essential lipoprotein of *B. subtilis* [116]. PrsA localizes to the outer surface of the cell membrane as distinct spots, arranged in a helical pattern of dimers or oligomers [107]. Although PrsA is indispensable for growth and cell viability, *lgt* and *lsp* mutants are still viable. This indicates that the unmodified and unprocessed forms of PrsA retain sufficient activity to sustain cell viability [38, 117]. PrsA belongs to the parvulin subfamily of peptidyl-prolyl cis/trans isomerases (PPIases) [118]. Interestingly, the PPIase domain is situated in-between the functionally relevant N- and C-terminal domains of PrsA [118]. The PPIase domain of PrsA exhibits PPIase activity and a possible chaperone activity *in vivo* at the membrane-cell wall interface [116]. Recently, it was confirmed experimentally that the stability and folding of several penicillin-binding proteins (PBP2a, PBP2b, PBP3 and PBP4) involved in the biosynthesis of the lateral cell wall are directly or indirectly

dependent on PrsA [107]. In addition to this, overexpression of PrsA was shown to enhance the secretion of several extracellular proteins, including α-amylases, in *B. subtilis* and *Lactococcus lactis* [53, 116, 119]. Furthermore, PrsA overproduction in *B. subtilis* resulted in 1.5-fold increased secretion and activity of the pharmacologically relevant human interferon-β (hIFN-β) with the AmyE propeptide [23].

Extracellular and membrane-bound proteases of *B. subtilis*

B. subtilis produces at least eight extracellular proteases at the end of the exponential phase of growth in liquid culture [120]. These have been identified as the alkaline serine protease subtilisin (AprE) [121, 122], the neutral protease NprE [123], the minor extracellular protease Epr [124, 125], the bacillopeptidase F (Bpr) [126], the Vpr protease [127], the metalloprotease Mpr [128, 129], the neutral protease NprB [123], and the cell wall-associated extracellular protease WprA [130] (Figure 3). Proteomics and transcriptomics studies have shown the involvement of the DegS-DegU two-component regulatory system in the expression of several of these exoproteases [108, 131, 132].

The extracellular proteases are synthesized with Sec-type signal peptides and exported from the cytoplasm via the Sec pathway. Although a potential RR-motif (KR) is present in the signal peptide of WprA, there is currently no published evidence for Tat-dependent WprA export under standard laboratory growth conditions. AprE and NprE are known to contribute 95% of the extracellular protease activity while the remaining activity is fulfilled by Epr, NprB, Bpr, Vpr, WprA and Mpr [133]. Extracellular proteases in *B. subtilis* are generally synthesized as inactive 'preproenzymes' in the cytoplasm. The pre-peptide (i.e. the signal peptide) directs export from the cytoplasm. The propeptide of the exported proenzyme is essential for the proper post-translocational folding of the proteases. Upon membrane

translocation, the proenzymes are processed into their proteolytically active form by the removal of the propeptide either by autoprocessing or heteroprocessing [56], [134-137]. For example, the inactive form of subtilisin (AprE) is converted into the active form by intramolecular autoprocessing in the extracytoplasmic space [135, 137], and Bpr is involved in the processing of pro-Mpr [138]. The main biological function of secreted proteases is to supply the *B. subtilis* cells with peptides and amino acids by the degradation of extracellular proteins from dead organic matter in the soil. Interestingly, these enzymes also impact on cell wall biogenesis since the stability of the cell wall lytic enzymes LytE and LytF is controlled by the cell wall-bound protease WprA and the extracellular protease Epr [139]. Also, the cell wall protein of unknown function WapA is a target of extracellular proteases [140]. Epr provides signals for the swarming motility of *B. subtilis* [141]. Lastly, extracellular proteases impact on the maturation of bacteriocins and signaling molecules. Thus, the processing of the lantibiotic subtilin of *B. subtilis* ATCC 6633 is achieved by AprE, WprA and Vpr [142]. Likewise, the minor extracellular proteases Epr and Vpr of *B. subtilis* are involved in processing of the Phr pentapeptide proCSF into the mature CSF that is active in extracellular signaling [143].

The extracellular proteases of *B. subtilis* and related bacilli are also very relevant for commercial applications. In particular, these enzymes are employed in the manufacture of detergents, tanning of leather, management of industrial and house hold wastes, bioprocessing of X-ray or photographic films for the recovery of silver, protein hydrolysate preparation in the food industry, synthesis of aspartame, and other applications [144]. Recently the fibrinolytic activity of Vpr was discovered. Accordingly, Vpr has the potential to work as a thrombolytic agent in medical applications [145, 146].

Notably, extracellular proteases are not only beneficial for industrial applications. Their downside is that they represent major bottlenecks during the production of secreted heterologous proteins in *B. subtilis*. This relates to the fact that such heterologous proteins often fold more slowly upon membrane translocation than native proteins of *B. subtilis*, or that the heterologous proteins expose cleavage sites that are recognized by particular secreted proteases. To overcome these bottlenecks, various single or multiple protease mutants were constructed, including the well-known strains WB600, lacking six protease genes, and WB800 lacking eight protease genes. Such strains can be employed for the enhanced production of α-amylase, artificial fusion lipase, streptavidin and many more proteins of commercial importance [147, 148].

The HtrA and HtrB proteases, which belong to the CssRS regulon, are also known to affect the secretion of heterologous proteins [149]. These proteases are predicted to be integral membrane proteins. As shown by immuno-fluorescence microscopy, HtrA and HtrB are randomly distributed as foci all over the cell surface. Interestingly, the HtrB foci outnumber the HtrA foci [150]. HtrA and HtrB are known to be involved in the quality control of secreted proteins by their protease and chaperone functions [151, 152]. Overproduction of a heterologous protein, like the α-amylase AmyQ of *Bacillus amyloliquefaciens*, is sufficient to induce so-called secretion stress. This secretion stress is most likely caused by the accumulation of misfolded AmyQ in the cell wall and at the cell wall-membrane interface, which is sensed by the CssRS two-component regulatory system (Figure 3). The CssRS two-component system then induces the expression of the *htrA* and *htrB* protease genes resulting in AmyQ degradation by HtrA and HtrB. Both proteases are known to be bottlenecks also in the (over-) production of certain membrane proteins [149, 153, 154]. Interestingly, the HtrA protease of the

pathogen *S. aureus* assists in the folding and maturation of surface components of the Agr-system [155].

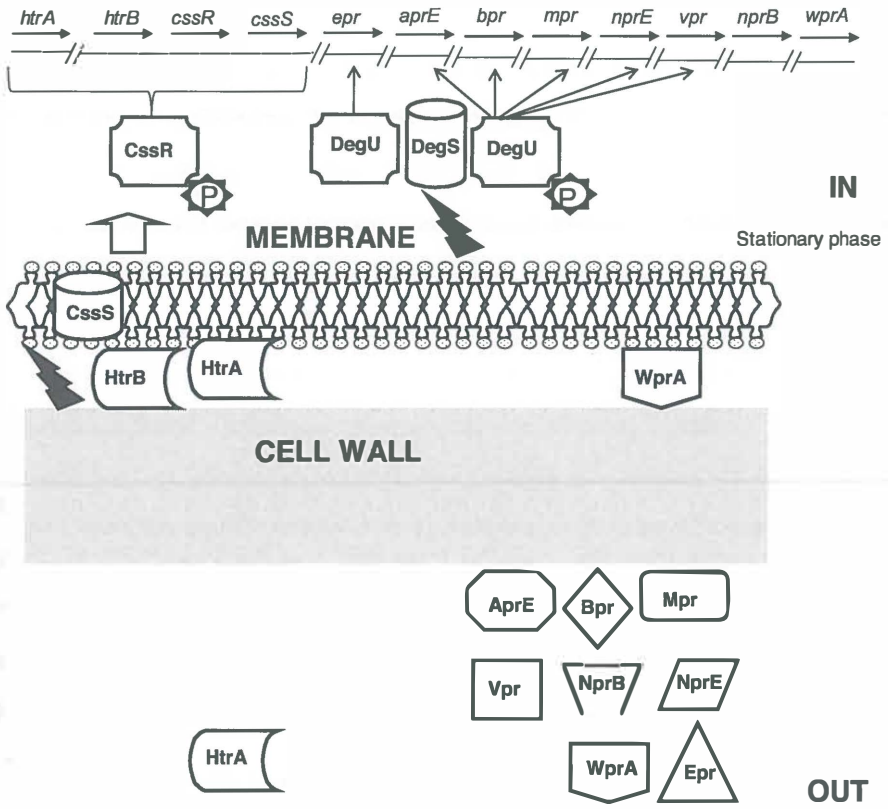

Figure 3. Schematic representation of the subcellular localization of the 10 extracytoplasmic proteases of *B. subtilis* addressed in this thesis. The key regulatory two-component systems CssRS and DegSU for the expression of the respective protease genes are also shown. For further information on the different proteases and regulatory systems, please see the main text.

Other extracytoplasmic proteases of *B. subtilis*

The bacterial cell membrane acts as a permeability barrier, which is critically important for the maintenance of cellular homeostasis [156]. Accordingly, membrane proteins are in charge of key processes like the transduction of signals, nutrient uptake, cell growth, excretion of metabolites, secretion of proteins and maintenance of the cell membrane integrity. Extensive membrane proteome analyses have been conducted and Otto *et al.* have identified 344 proteins in the membrane fraction as membrane proteins [157-160]. In addition, prediction algorithms for membrane proteins suggest that there are probably between 1050 and 1150 integral membrane proteins in *B. subtilis* [161]. Like proteins in the cytoplasm and cell wall, these membrane proteins can be targets for proteolysis. Several proteases for membrane protein turnover have been identified in *B. subtilis*. These include FtsH, which is homologous to the well-studied FtsH protein of *E. coli* and the mitochondrial m-AAA proteases [156, 162, 163]. FtsH of *B. subtilis* is an ATP- and Zn^{2+}-dependent metalloprotease localized in the cell membrane. FtsH mainly targets cytoplasmic and inner membrane proteins for degradation. The best-known FtsH substrates are the Spo0E and Spo0M [156, 164, 165]. Furthermore, the membrane proteases PrsW and RasP are known to be involved in the regulated intramembrane proteolysis of the membrane protein RsiW. The RsiW protein is an anti-sigma factor that sequesters the SigW sigma factor. Upon degradation of RsiW by PrsW and RasP, the SigW protein is released into the cytoplasm, which triggers the expression of the SigW stress regulon [156, 166, 166, 167].

Proteomics analyses of extracellular protease function in *B. subtilis*

Several *B. subtilis* mutants lacking extracellular proteases have been subjected to proteomics analyses. These include a *wprA* single mutant, a *wprA epr* double mutant, a mutant lacking seven extracellular proteases (WB700) and a mutant lacking eight extracellular proteases (WB800) [140, 168]. These analyses indicated that cell wall-binding proteins like WapA and YvcE are the substrates of extracellular proteases. Particularly Epr is considered as a major protease involved in the generation of extracellular WapA processing products. In addition, the absence of extracellular proteases resulted in a stabilization of the WapA processing product CWBP105 [140]. Analysis of the extracellular proteome of the WB800 strain showed that a substantial number of extracellular proteins are subject to proteolysis, including several proteins with signal peptides (e.g. YjcM, YocH, YqxI, YxaL, and YybN), the membrane-bound and extracellular protease HtrA, the flagellar proteins FlgB, FlgC, FlgE, FlgK, FlhO, FlhP and FliD, as well as some cytoplasmic proteins (Tig and YbdO) [168].

The presence of either HtrA or HtrB appeared to be very important for the survival of *B. subtilis* since it was difficult to obtain an *hrtA htrB* double deletion mutant. Therefore exoproteome analyses were performed with *B. subtilis* strains lacking either *htrA* or *htrB*, or a double mutant with an unidentified suppressor mutation. Interestingly, these exopreoteome analyses indicated that a conserved chaperone activity of HtrA is required for the stabilization of the secreted YqxI protein. Over all the exoproteome analyses of *B. subtilis* strains lacking extracellular and/or membrane-bound proteases turned out very helpful in the identification of the substrates that they degrade and the possible chaperone function of HtrA [152].

Aims and scope of this thesis

As outlined in the previous paragraphs, extracytoplasmic proteases have a major impact on the stability and quality of secreted proteins, secreted heterologous proteins in particular. Therefore, the research described in this thesis was aimed at obtaining a better understanding of the roles of extracytoplasmic proteases of *B. subtilis* in the quality control of exported proteins and the overall biogenesis and composition of the exoproteome. For this purpose, a collection of strains deficient in the production of multiple extracellular, cell wall-bound and membrane-bound proteases were studied. Of special relevance for the present studies was the use of multiple protease mutant strains that also lacked HtrA, HtrB, or both of these quality control proteases.

Chapter 1 of this thesis gives an introduction to the secretome of *B. subtilis*. It summarizes the mechanisms of protein export from the cytoplasm and their relevance in the production of secreted homologous and heterologous proteins. Special attention is dedicated to the extracellular proteases of *B. subtilis*, and to several important exported proteins that were identified in the present studies as substrates for proteolysis. The latter applies specifically to the secreted Dyp-type peroxidase YwbN, which is exported via the Tat translocation machinery, and to the folding catalyst for exported proteins PrsA.

Chapter 2 describes an in-depth comparison of the exoproteomes of two *B. subtilis* strains deficient in multiple extracytoplasmic proteases that was performed following a combined gel electrophoresis, liquid chromatography and mass spectrometry (GeLC-MS) approach. The investigated strains lacked either AprE, Bpr, Epr, Mpr, NprB, NprE, Vpr and WprA (i.e. strain

BRB08), or these eight extracytoplasmic proteases plus the two membrane-bound proteases HtrA and HtrB (i.e. strain BRB14). The GeLC-MS data indicate that various secreted proteins, lipoproteins and membrane proteins of *B. subtilis* are degraded by this organism's own extracytoplasmic proteases. Furthermore, the exoproteome data suggest that HtrA and HtrB are involved in the degradation of particular membrane proteins, and that these two quality control proteases are very important for maintaining the integrity of the cell membrane.

Chapter 3 discusses the role of extracellular proteases towards the quality control and degradation of the Tat substrate YwbN. As pointed out in Chapter 1, previous research has shown that bacterial Tat translocases can export fully folded proteins from the cytoplasm. Such proteins are usually resistant to proteolysis. Since proteolysis is an important theme in *B. subtilis*, as was underscored by the studies described in Chapter 2, the question was addressed whether the Tat substrate YwbN might also be subject to proteolysis. The results of these studies show that multiple extracellular proteases, including AprE, Bpr, NprE, Vpr, WprA, HtrA and HtrB degrade YwbN. Thus, it seems that YwbN is a substrate for both feeding and quality control proteases. These findings challenge the hypothesis that Tat-dependently secreted proteins of *B. subtilis* are protease-resistant. Instead, the present observations suggest that YwbN is either not fully folded upon membrane translocation, cell wall passage and secretion into the growth medium, or that the folded YwbN exposes protease cleavage sites.

Chapter 4 reports on the role of extracytoplasmic proteases in the degradation of extracytoplasmic catalysts for protein folding in *B subtilis*. As shown in chapter 2, the deletion of multiple genes for extracytoplasmic proteins resulted in the secretion of many proteins at elevated levels. This

most likely relates to the degradation of these proteins by one or more of the eliminated proteases, but indirect effects are also conceivable. Therefore, it was investigated whether the deletion of multiple proteases would impact on the post-translocational folding catalyst PrsA and the membrane-bound quality-control factors HtrA and HtrB. Interestingly, the results show that PrsA, HtrA and HtrB are substrates of multiple extracytoplasmic proteases. Thus, the improved secretion of proteins by multiple protease mutant strains may relate not only to reduced proteolysis, but also to elevated levels of chaperones and quality control factors.

Chapter 5 summarizes the main conclusions of the research documented in this thesis. This chapter also presents an outlook on the possibilities for future fundamental and applied research on the proteolysis of extracytoplasmic proteins in *B. subtilis*.

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Chapter 2

Extracytoplasmic proteases determining the 'life and death' of secreted proteins, lipoproteins and membrane proteins in *Bacillus subtilis*

Chapter 2: Extracytoplasmic proteases determining 'life and death' of secreted proteins, lipoproteins and membrane proteins in *Bacillus subtilis*

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Submitted

Abstract

Gram-positive bacteria are known to export many proteins to the cell wall and growth medium and, accordingly, many studies have addressed the respective protein export mechanisms. In contrast, very little is known about the subsequent fate of these proteins. The present studies were therefore aimed at determining the fate of native exported proteins in the model organism *Bacillus subtilis*. Specifically, we employed a GeLC-MS approach to distinguish the roles of the membrane-associated quality control proteases HtrA and HtrB from those of eight other proteases that are present in the cell wall and/or growth medium of *B. subtilis*. Notably, HtrA and HtrB were previously shown to counteract potentially detrimental 'protein export stresses' upon overproduction of membrane- or secreted proteins. Our results show that many secreted proteins, lipoproteins and membrane proteins of *B. subtilis* are potential substrates of extracytoplasmic proteases. Moreover, potentially important roles of HtrA and HtrB in the folding of native secreted proteins into a protease-resistant conformation, the liberation of lipoproteins from the membrane-cell wall interface, and the degradation of membrane proteins are uncovered. Altogether, our observations show that HtrA and HtrB are crucial for maintaining the integrity of the *B. subtilis* cell even under non-stress conditions.

Introduction

Gram-positive bacteria are known to secrete a wide range of proteins with different biological functions and enzymatic activities into their extracellular milieu. Especially the secreted enzymes of *Bacillus* species are of biotechnological relevance and therefore many studies have addressed their secretion mechanisms. In contrast, relatively little is known about the fate of the secreted proteins after they have served their purpose. In natural environments, these proteins may diffuse into the environment where they can end up as a source of peptides and amino acids for other organisms. However, also the cells producing the secreted proteins may benefit from these proteins after they are no longer actively performing their physiological function, for example by degrading these proteins and recycling the degradation products. The extent to which this degradation happens is currently not known, which is surprising in view of the high scientific interest in regulated protein turnover and the high commercial value of many secreted enzymes.

Bacillus subtilis is one of the best-studied Gram-positive bacteria that is capable of secreting high amounts of proteins into its growth medium. Due to this high secretion capacity *B. subtilis* is widely used for enzyme production, especially in the detergent industry [1] . There is also strong interest in the use of *B. subtilis* for the production of secreted heterologous proteins, but this is often hampered by quality control processes in the membrane and cell wall, as well as substantial proteolytic activity in the growth medium [2]. This problem has focused attention on the proteases involved in the degradation of heterologous proteins [3-6] .

The majority of proteins secreted by *B. subtilis* are exported from the cytoplasm via the Sec pathway, which facilitates their membrane translocation in an unfolded state [7]. Accordingly, the translocated proteins need to fold into their active and protease-resistant conformation after the membrane passage is complete [8]. This folding process is guided by membrane-associated folding catalysts, such as the lipoprotein PrsA [9, 10], which has peptidyl-prolylcis/trans isomerase activity, and the membrane proteins BdbC and BdbD, which have thiol-disulfide oxidoreductase activity [11-14]. Propeptides and metal ions also play significant roles in folding of secreted proteins [8]. Propeptides can be regarded as intramolecular chaperones that help the respective proteins, including many *Bacillus* proteases, to achieve their fully folded conformation [15, 16]. Metal ions, such as Ca^{2+}, Fe^{3+} and Mg^{2+}, are known to enhance the folding and stability of many secreted proteins of *B. subtilis* [8, 17-19]. The quality of the folding process of translocated proteins is monitored by the CssR-CssS two-component regulatory system of *B. subtilis* [20-22]. If malfolded proteins accumulate at the membrane-cell wall interface, CssRS induces the expression of the membrane-associated HtrA and HtrB proteins that have both extracytoplasmic protease and chaperone activities [20, 23, 24] . Intriguingly, only one secreted protein of *B. subtilis* (YqxI) was so far shown to require the potential chaperone activity of HtrA for folding into a stable conformation [18]. Based on its key role in the upregulation of HtrA and HtrB, the CssRS system is regarded as a major bottleneck in the overproduction of exported proteins [5, 25]. It should be noted that the CssRS system is also induced if either HtrA or HtrB is absent, suggesting that the low basal level of expression of these proteins is

important for preventing the accumulation of malfolded native proteins at the membrane-cell wall interface of *B. subtilis* [26-28]. Notably, the relevance of this low-level expression of HtrA and HtrB in non-'secretion-stressed' cells was so far not experimentally assessed.

The second major bottleneck in the secretion of heterologous proteins is formed by a highly potent cocktail of eight secreted proteases, namely AprE, Bpr, Epr, Mpr, NprB, NprE, Vpr, and WprA [2]. AprE and NprE account for ~95% of the total extracellular proteolytic activity, but the activity of the other six proteases is sufficient to set major limits to heterologous protein production [29]. In this respect, WprA is especially notorious since substantial amounts of this protease are retained in the cell wall, degrading unfolded proteins that emerge from the Sec translocon [3, 30, 31]. Interestingly, previous studies with multiple protease mutant strains provided some evidence that the secreted proteins do not only degrade heterologous proteins, but also certain native cell wall-associated or secreted proteins. For example, this concerned the cell wall-binding proteins WapA and YvcE [32]. Furthermore, WprA can degrade a mutant form of the signal peptidase SipS at the membrane-cell wall interface [31] and, together with Epr, WprA is involved in degradation of the wall-bound autolysins LytE and LytF [33]. It should be noticed that the turnover of exported proteins is likely to be beneficial for the cells in many respects. Firstly, malfolded proteins accumulating at the membrane-cell wall interface can interfere with essential processes that are needed for membrane and/or cell wall integrity [20]. Secondly, limiting the levels of autolysins is likely to be important for maintaining a robust cell

wall. While autolysins are needed to expand the cell walls of growing cells and to separate cells that have divided [34], their function is no longer needed when cells enter the stationary phase where actually most of the proteases are produced [35]. Thirdly, the extracytoplasmic proteases have important functions in the processing of other exported proteins. For example, Vpr and WprA are involved in maturation of the lantibiotic subtilin [36]; AprE and Vpr are needed to process secreted Phr peptides for quorum sensing [37, 38], and Bpr is needed to process Mpr.

Altogether, the available data show overlapping functions of different exported proteases. This raised the question to what extent the activities of typical quality control proteases, such as HtrA and HtrB overlap with those of other extracytoplasmic proteases (schematically represented in Figure 1). The present studies were therefore aimed at dissecting the roles of these proteases by a detailed comparison of the exoproteome of a strain that lacks eight secreted proteases (BRB08) with the exoproteome of a strain that lacks the same eight proteases *plus* the membrane-bound proteases HtrA and HtrB (BRB14). The results reveal important roles of extracellular and membrane-bound proteases in the 'life and death' of secreted proteins, lipoproteins and multipass membrane proteins. Importantly, this allowed us to pinpoint proteins that potentially require the chaperone activities of HtrA and HtrB for folding.

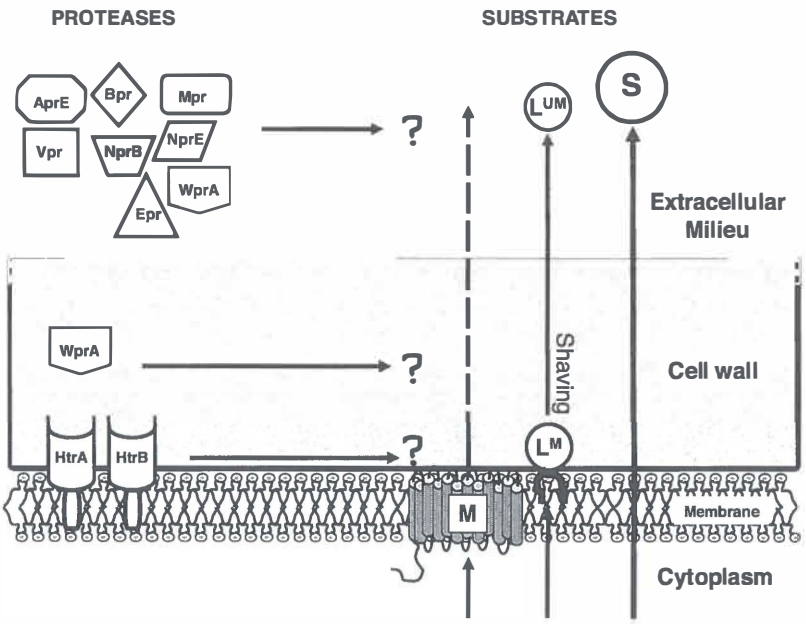

Figure 1. Schematic representation of extracytoplasmic proteases and their substrates in *B. subtilis*. *B. subtilis* produces at least ten extracytoplasmic proteases of which two are membrane-associated (HtrA and HtrB) and eight are secreted into the extracellular milieu (AprE, Bpr, Epr, Mpr, NprB, NprE, Vpr and WprA). In addition, WprA is also active at the membrane-cell wall interface. In the present studies we have investigated to what extent secreted proteins (S), lipoproteins (L) and membrane proteins (M) are substrates of these proteases. Notably, upon proteolytic 'shaving' of lipid-modified mature lipoproteins (L^M) from the membrane, unmodified mature lipoproteins (L^{UM}) are released into the growth medium.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. The strains were grown under vigorous agitation at 37°C in Lysogeny Broth (LB), which consisted of 1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.4.

Table 1. *B. subtilis* strains used in this study

Strains	Genotype	Reference
168	*trpC2*	[39]
BRB08	*trpC2 ΔnprB ΔaprE Δepr Δbpr ΔnprE Δmpr Δvpr ΔwprA*	Cobra Biologics
BRB14	*trpC2 ΔnprB ΔaprE Δepr Δbpr ΔnprE Δmpr Δvpr ΔwprA ΔhtrA ΔhtrB*	Cobra Biologics
lytC	*trpC2*; *pheA3*; *purAJ6*; *hisA35*; *metB5*; *lytC*::Cm; Cm^R	[40]
lytD	*trpC2*; *pheA3*; *purAJ6*; *hisA35*; *metB5*; *lytD*::Cm; Cm^R	[40]

BRB ***Bacillus*** **R**ecipharm cobra **B**iologics

Sample preparation

Pre-cultures were inoculated from overnight cultures to an OD_{600} of 0.1, propagated to an OD_{600} of 0.5, and were then used as inoculum for the main culture from which samples were taken for proteome analyses. Cells were separated from the growth medium by centrifugation (4000 ×*g*, 10 min, 4°C) two hours after entry into stationary phase. After filtration of the growth medium fraction (Millipore filters pore size 0.22 μm), proteins in the growth medium were precipitated with 10% w/v ice-cold TCA (4°C, overnight). The precipitated proteins were pelleted by centrifugation (13,000 ×*g* 20 min) and washed with ice-cold acetone. The dried protein pellet was dissolved in an 8M urea solution, and the protein concentration was determined with the Bio-Rad D_c^{TM} protein assay kit according to manufacturer's instruction. 20 μg of protein was diluted with LDS reducing sample buffer (Invitrogen) and incubated at room temperature for 30 min. The proteins were then separated on a 10% NuPAGE gel (Invitrogen) and subsequently stained with Coomassie Brilliant Blue [41].

To obtain crude cell envelope fractions, the collected cells were resuspended in PBS with protease inhibitor (Complete Mini, EDTA-free, Roche Diagnostics) and disrupted by bead beating (Bertin Technologies; 6,500 rpm, 30 s with 30 s pause). After centrifugation (13,000 ×*g* at 4°C) to remove unbroken cells and cellular debris, the resulting supernatant was subjected to ultracentrifugation (200,000 ×*g*, 30 min, 4°C) in order to separate the cytosolic proteins from the crude cell envelope fraction. The crude cell envelope fraction was then resuspended in an 8M urea solution and the protein concentration was determined.

Mass spectrometry

Proteome analyses involved a one-dimensional (1D) NuPAGE separation of protein samples (20 µg) followed by tryptic digestion and LC-MS analysis (GeLC-MS). In contrast to the traditional two-dimensional (2D) PAGE, the GeLC-MS approach allows the separation of proteins with extreme pI and high hydrophobicity, including membrane proteins. Briefly, after electrophoretic separation and gel staining, the gel lanes were cut into 10 equidistant pieces. These gel pieces were destained and washed with washing buffer (50 mM ammoniumbicarbonate, 30% ACN) and dried. Tryptic digestion was performed overnight at 37°C followed by elution of the tryptic peptides with water.

For LC-MS/MS measurements, the desalted tryptic digest was subjected to a reversed phase column chromatography run on an EASYnLC (Proxeon, Odense, Denmark). Emitter tips for the self-packed columns were prepared by pulling out tips using 100 µm i.d. fused silica capillaries with an o.d. of 360 µm with a laser puller (P2000, Sutter Instruments, USA). These emitter tips were packed at >200 bar using a slurry of C18 material (Luna 3µ C18(2) 100A, Phenomenex, Germany) in methanol attached to a pressurized cell. After packing, the columns were equilibrated with the loading buffer (0.1% acetic acid). Self-packed columns with a length of 30 cm were used in an open vented one-column setup with a loading volume of 10 µl at a flow of 700 nl/min at a maximum of 220 bar and a subsequent flow rate of 300 nl/min. Separation of the peptides was achieved by the application of a binary non-linear 70-min gradient from 5-50% ACN in 0.1% acetic acid. The self-packed

columns were mounted in a modified nano-electrospray ion source with liquid junction of the voltage (2,400V) applied between orifice and emitter tip.MS and MS/MS data were acquired with the LTQ-Orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany). After a survey scan in the Orbitrap (r= 30,000), MS/MS data were recorded for the five most intensive precursor ions in the linear ion trap. Singly charged ions were not taken into account for MS/MS analysis. The lock mass option was enabled throughout the analysis. The mass spectrometric data was then subjected to database searching via Sorcerer-Sequest. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version v.27, rev. 11). Sequest was set up to search a *B. subtilis* target-decoy protein sequence database that included the complete proteome set of *B. subtilis* extracted from UniprotKB release 12.7 [42], and a set of common laboratory contaminants compiled with Bioworks Browser (Thermo Fisher Scientific, San Jose, CA, USA), assuming the digestion enzyme trypsin. Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a search tolerance of 10 ppm for the overview scans. Oxidation of methionine was specified in Sequest as a variable modification. Scaffold (version Scaffold_3_00_04, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded Xcorr values of 2.2/ 3.5/ 3.75 for doubly/ triply and quadruply charged ions. A protein was regarded as significantly identified when at least two peptides per protein were identified in at least three of four biological replicates. These criteria resulted in

no false positive identifications. All MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [43] with the dataset identifier PXD000078. All peptide identifications are listed in Supplementary Table S1 (available on request). It should be noted here that tryptic cleavage of YomL and YddT generates similar peptides. However, the cleavage of YomL results in two unique 'diagnostic' peptides that proves the presence of YomL in the respective samples.

Western blotting

For Western blotting analyses, proteins were separated on 10% NuPAGE gels. To this end, the gels were loaded with 2.5 µg of the cell envelope fractions, or 20 µg of secreted proteins. The separated proteins were semi-dry blotted (75 min at 1 mA / cm^2) onto a nitrocellulose membrane. Subsequently, PrsA, FeuA, YclQ or BdbD were detected with specific polyclonal antibodies raised in rabbits. Visualization of bound antibodies was performed using IRDye 800 CW goat anti-rabbit secondary antibodies in combination with the Odyssey Infrared Imaging System (LiCor Biosciences). Fluorescence was recorded at 800 nm.

Zymography

Zymography was performed as described previously [40, 44] with a few alterations. *B. subtilis* was grown in 100 ml LB broth until 2 h after entry into the stationary growth phase. Cells were collected by centrifugation and subsequently disrupted by sonication (Misonix ultrasound liquid processor S-4000) at an amplitude of 25% for 1

min with 30 s intervals. Samples with 70 μg of cell extract were loaded on SDS-PAGE gels containing 0.1 % *Micrococcus* cell wall (Sigma-Aldrich). Upon electrophoresis, autolysin activity was measured in the gels after renaturation of the proteins.

Results and Discussion

The exoproteomes of multiple protease-deficient *B. subtilis* strains

To assess the composition of the exoproteomes of strains BRB08 and BRB14, which respectively lack 8 or 10 extracytoplasmic proteases, a 1D NuPAGE analysis was performed. As shown in Figure 2, the complexity of the exoproteome patterns of the protease mutant strains as reflected by 1D NuPAGE was much higher than that of the parental strain 168. This result was anticipated on the basis of previous studies with *B. subtilis* strain WB800 [6], which lacked the same extracellular proteases as strain BRB08, the main difference being that all protease genes were completely deleted from strain BRB08 and that no antibiotic resistance markers were left in the genome of this strain. Due to the high complexity of the BRB08 and BRB14 exoproteome samples, no obvious differences were detectable upon visual inspection of the protein banding patterns (Figure 2). Notably, upon 1D NuPAGE, samples of the parental strain 168 appeared to contain less protein than samples of the protease mutants BRB08 and BRB14 even though equal amounts (20 μg) were loaded on the gel. This suggests that samples of the parental strain 168 contained a relatively large amount of

small proteins and peptides that were detectable on the gel due to their high electrophoretic mobility.

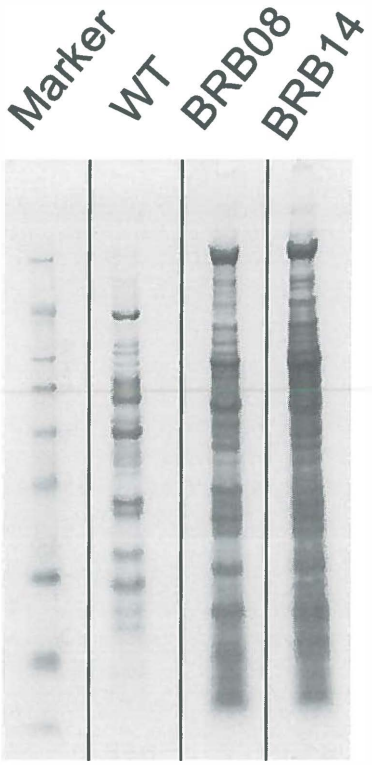

Figure 2. Exoproteome profiles of strains lacking multiple extracytoplasmic proteases. The *B. subtilis* strains 168 (wt), BRB08 (08) and BRB14 (14) were grown in LB broth till 2 h post-exponential growth phase. Cells were separated from the growth medium by centrifugation. Proteins in the growth medium fractions were precipitated with 10% TCA and separated by NuPAGE. The separated proteins were then stained with colloidal coomassie. The depicted gels *plus* three additional biological replicates derived from each individual strain were subsequently used for GeLC-MS analyses.

Therefore, possible differences in the exoproteomes of strains BRB08, BRB14 and the parental strain 168 were further examined by GeLC-MS. This resulted in the identification of 686 proteins (Supplementary Table S2; available on request) of which 138 were common to the exoproteomes of the three investigated strains. Importantly, the GeLC-MS analyses identified substantial differences in the exoproteome composition of the investigated strains, as only 18 proteins were unique to the parental strain 168 and 22 proteins to strain BRB08, whereas 227 proteins were exclusively identified in the samples derived from strain BRB14 (Figure 3).

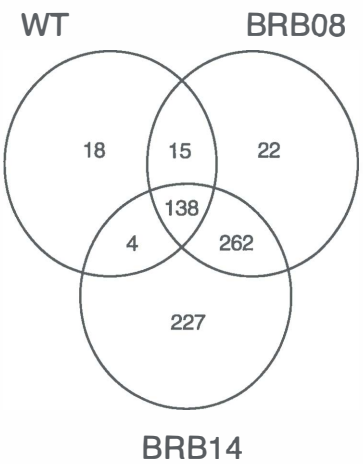

Figure 3. Comparison of proteins identified in the exoproteomes of multiple protease mutants and their parental strain. Proteins in exoproteome samples of the *B. subtilis* protease mutants BRB08 (08) and BRB14 (14) or the parental strain 168 (wt) were identified by GeLC-MS analyses (Table S2). The Venn diagram shows the numbers of proteins that are either unique for each strain or common for two or three strains.

The identified proteins were evaluated regarding their predicted subcellular localization using the LocateP algorithm [42]. This revealed that 12% of the proteins identified in the exoproteome sample of the parental strain were predicted to be located in the cytosol while this percentage was increased to 45% and 74% for strains BRB08 and BRB14, respectively (for details, see Table S2). To verify this finding, we compared the levels of the cytosolic marker protein Thioredoxin A (TrxA) in the culture medium by Western blotting. As shown in Figure 4A, the obtained signal for TrxA was 2.5- and 6.5-fold more intense in the exoproteome samples of strains BRB08 and BRB14, respectively, than in the exoproteome sample of the parental strain. Thus, the accumulation of TrxA in the culture medium is well correlated to the percentage of cytosolic proteins in the culture medium as determined by GeLC-MS (Figure 4B). Several explanations are conceivable for the increased levels of cytoplasmic proteins in the growth media of the protease-deficient *B. subtilis* strains. Firstly, this might be due to a reduced level of autolysin degradation, which would result in increased cellular lysis [45]. Secondly, cytoplasmic proteins liberated by cell lysis might accumulate in the growth medium due to lack of extracellular protease activity [6]. On top of that, additional cell lysis of the BRB14 strain could relate to a lack of quality control activity at the membrane-cell wall interface due to the absence of HtrA and HtrB.

To determine whether the cells of strains BRB08 and BRB14 might contain elevated autolysin levels, a zymogram analysis of autolysin activity was performed (Figure 4C). A significant clearing zone corresponding to a high molecular weight

autolysin was observed only in the cell fractions of strains BRB08 and BRB14. The appearance of this zone correlated with the disappearance of a lower molecular weight form of LytD (~30kDa; labeled $LytD^{L}$) and hence the high molecular autolysin is most likely an unprocessed from of LytD (~95 kDa; labeled $LytD^{H}$). Notably, this $LytD^{H}$ activity represented the first clearing zone detectable during the zymography, and it thus reflected the strongest cell wall hydrolytic activity in the BRB08 and BRB14 strains (not shown). These findings show that one or more of the proteases lacking from strain BRB08 are involved in the cleavage of LytD, which is synthesized in a pro-form of about 95 kDa. Furthermore, it seems that at least the activity of LytD is enhanced in the absence of these proteases, which suggests that increased autolysin activity could be a determinant in the release of cytoplasmic proteins from strains BRB08 and BRB14 into the growth medium.

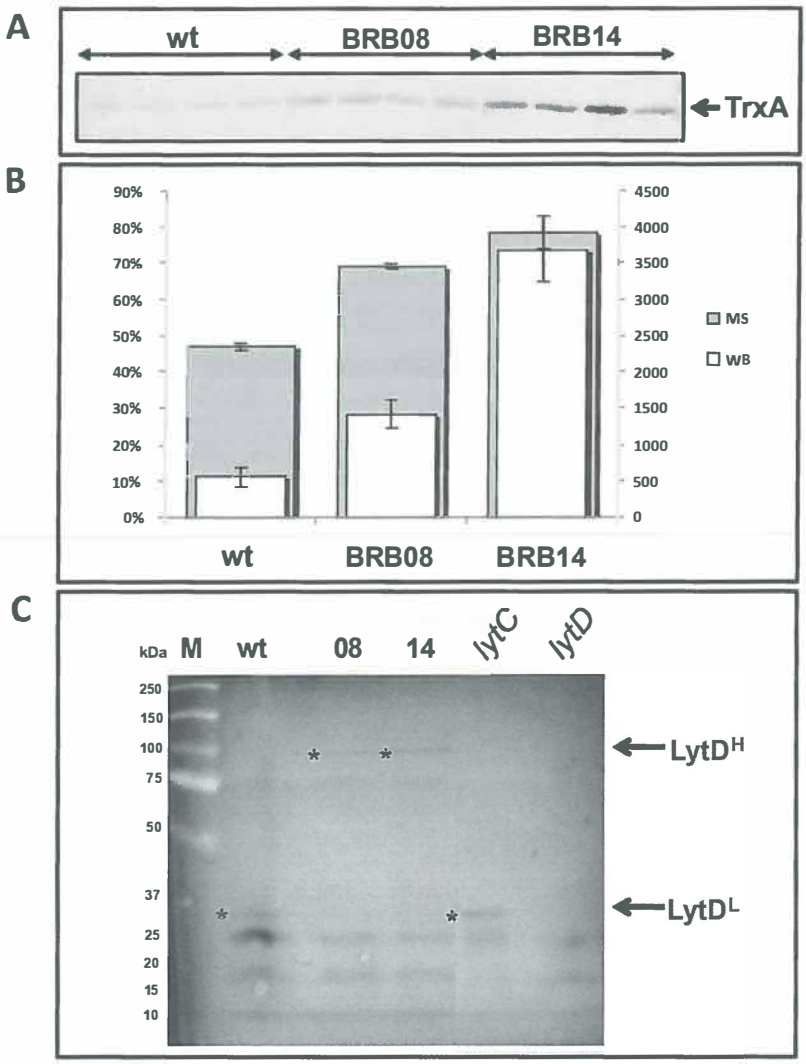

Figure 4. Integrity assessment of protease mutants and their parental strain. *B. subtilis* cells were cultured as described for Figure 2. **A.** Western blotting analysis of the amounts of the lysis marker protein TrxA in growth medium fractions of the *B. subtilis* strains 168 (wt), BRB08 (08) and

BRB14 (14). Gels were loaded with 10 μg of protein isolated from the respective growth media. Note that TrxA is normally encountered in the cytoplasm of *B. subtilis* where it acts as the major thioredoxin [13]. **B.** Comparison of the percentages of cytoplasmic proteins in exoproteome samples identified by GeLC-MS (grey bars, left y-axis) with the amounts of the cytoplasmic marker protein TrxA in the respective samples (white bars, right y-axis; indicated in arbitrary units). The amounts of TrxA in each sample were quantified by Western blotting and subsequent image analysis using ImageJ. **C.** Zymogram analyses of autolysin activity in cells of *B. subtilis* strains 168 (wt), BRB08 (08), BRB14 (14), *lytC* and *lytD*. The positions of high- and low molecular weight forms of LytD are indicated with $LytD^{H}$ and $LytD^{L}$, respectively. The respective bands are furthermore highlighted with *. Note that an inverted (negative) image of the zymogram is shown.

Impact of protease mutations on secreted proteins

In total 63 proteins with typical signal peptides were identified during our MS analyses of which 27 were common to the exoproteomes of the three analyzed strains (Figure 5, Table S2). The fact that the remaining 36 proteins were undetectable in the exoproteomes of one or two strains suggests that the presence or absence of certain proteases influenced their appearance in the extracellular proteome. In this context, it is however important to bear in mind that, while MS is an extremely powerful technology for proteome analyses, it is one of the main limitations of MS that the lack of detection of a particular protein may not always mean this protein was completely absent from an analyzed sample. Therefore, to minimize possible misinterpretations of our MS-based exoproteome analyses, samples from four biological replicates were analyzed for all three investigated strains.

Specifically, five secreted proteins, namely LipB, YbbC, YjdB, YraI and YwmC were unique to the exoproteome of strain BRB08. This implies that these five proteins are potential substrates of the eight extracytoplasmic proteases that are absent from strain BRB08. Intriguingly, LipB, YbbC, YjdB, YraI and YwmC were also absent from strain BRB14, which may suggest that they require the chaperone activity of HtrA and/or HtrB for folding into a protease-resistant conformation. If so, this means that in the absence of HtrA and HtrB these secreted proteins might undergo proteolysis by one or more as yet unidentified proteases. Nine additional secreted proteins were common to the exoproteomes of the parental strain 168 and strain BRB08 (i.e. BglC, CotN, MsmE, SacC, YdaJ, YdhT, YvfO, YxiA and YwoF), but absent from the exoproteome of strain BRB14 (Figure 5). This suggests that these proteins may also require the chaperone activities of HtrA and/or HtrB for their post-translocational folding. However, once folded they would be resistant to the extracytoplasmic proteases of *B. subtilis*. Only one protein was unique to the exoproteome of the BRB14 strain. This concerned YonN, which is secreted via an ABC transporter according to LocateP predictions. Accordingly, YonN might be a substrate for degradation by HtrA and/or HtrB. However, it should be noted that our present proteomics analyses do not allow a clear distinction between gene regulatory and post-transcriptional effects. Therefore, at least some of the observed effects described here in the following sections may relate to transcriptional regulation in response to stresses caused by the absence of multiple proteases.

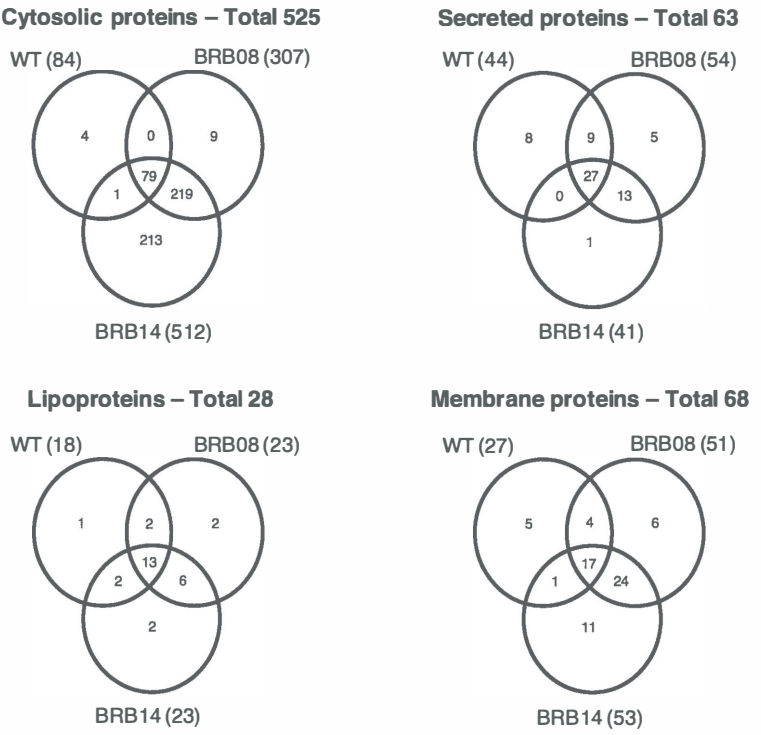

Figure 5. Comparison of the cytoplasmic proteins, secreted proteins, lipoproteins and membrane proteins identified in the exoproteomes of multiple protease mutants and their parental strain. The proteins that were identified by GeLC-MS analyses of exoproteome samples of the *B. subtilis* strains 168 (wt), BRB08 (08) and BRB14 (14) were compared in relation to their subcellular localization as predicted by the LocateP algorithm. The Venn diagrams show the numbers of proteins that are either unique for each strain, or common for two or three strains. The total numbers of proteins identified for each group (cytosolic, secreted, lipo-or membrane proteins) are indicated above each diagram, and the total numbers of such proteins identified for each strain are indicated in parentheses next to the strain name.

Impact of protease mutations on lipoproteins

Altogether 28 known or predicted lipoproteins were identified during our MS analysis of exoproteome fractions (Figure 5, Table S2). Thirteen of these lipoproteins, namely ArtP, FeuA, FhuD, MntA, OppA, PbpC, TcyA, YclQ, YerH, YfiY, YfmC, YhfQ and YxeB were common to *B. subtilis* 168 and the BRB08 and BRB14 strains. We therefore propose that these lipoproteins are not major targets of the extracytoplasmic proteases addressed in the present studies. Notably, the lipid-modification of lipoproteins serves to retain these proteins at the membrane-cell wall interface. However, as shown by N-terminal amino acid sequencing, in *B. subtilis* and other Gram-positive bacteria, proteolytic removal of the lipid-modified N-terminal cysteine residue from mature lipoproteins can occur, resulting in the release of the unmodified mature lipoprotein into the growth medium [47]. This process which occurs after signal peptide processing by the lipoprotein-specific signal peptidase II (LspA) has been referred to as proteolytic 'lipoprotein shaving' [46, 47]. Notably, as shown the lipoprotein PenP of *Bacillus licheniformis*, lipoprotein shaving may also relate to the removal of the lipid-modified N-terminal cysteine plus some additional residues of the mature lipoprotein [48]. Since the lipoproteins ArtP, FeuA, FhuD, MntA, OppA, PbpC, TcyA, YclQ, YerH, YfiY, YfmC, YhfQ and YxeB were common to the exoproteomes of all three strains investigated in the present studies, we can conclude that none of the ten proteases that were deleted from strain BRB14 are strictly required for the shaving of these lipoproteins. Nevertheless, as shown by Western blotting, FeuA accumulation in the medium was enhanced in strain BRB14 (Figure 6), suggesting that HtrA and/or

HtrB might be involved in its liberation from the cell, or that FeuA may be degraded to some extent by these quality control proteases. The latter possibility would be supported by the observation that the relative amounts of an extracellular FeuA degradation product are reduced in strain BRB14 (Figure 6; compare the two FeuA bands in the growth medium fractions). This underscores the view that our MS analyses of the exoproteomes of strains BRB08 and BRB14 are qualitative rather than quantitative. Western blotting analyses revealed a somewhat different behavior for the lipoprotein YclQ, which was detected in the form of two distinct bands in medium samples of the parental strain 168 and strain BRB08 (Figure 6). The lower molecular weight band was absent from the medium sample of strain BRB14 and the amount of the higher molecular weight band was reduced in this sample. These findings suggest that HtrA and/or HtrB may have a role in the conversion of the high molecular weight band to the smaller form. A possible role of these two proteases in YclQ shaving and degradation is also suggested by the elevated levels of the high molecular weight "isoform" of this lipoprotein in the cell envelope of strain BRB14.

Interestingly, the lipoproteins DppE and YtcQ were identified in the exoproteomes of strains 168 and BRB08, but not in the exoproteome of strain BRB14. This indicates that these lipoproteins are potentially released into the growth medium through the action of HtrA and/or HtrB. Furthermore we identified two lipoproteins, YurO and YxiP, as being unique for the exoproteome of strain BRB08. This suggests that the latter two lipoproteins may be released from the cell by either HtrA or HtrB. Upon release from strain BRB08, YurO and YxiP are apparently

stably maintained in the exoproteome. On the other hand, YurO and YxiP are not detectable on the exoproteome of the parental strain 168, which suggests that they are substrates for degradation by one or more of the eight proteases that are absent from strain BRB08. Altogether, we conclude that DppE, YtcQ, YurO and YxiP are most likely 'shaved' from the membrane surface by HtrA and/or HtrB.

Six lipoproteins were common to the exoproteome of strains BRB08 and BRB14, namely AppA, PrsA, RbsB, YcdA, YjhA and YvrC. This implies that, once they have been released from the cell through shaving by an unidentified protease, these lipoproteins are most likely degraded by one or more of the extracytoplasmic proteases lacking from strain BRB08. Consistent with the MS data, PrsA was detected by Western blotting only in the growth medium fractions of the BRB08 and BRB14 strains albeit that the levels were higher in the medium of strain BRB14 (Figure 6). Furthermore, substantially elevated levels of PrsA were identified in the cell envelope fractions of the two protease mutant strains. These findings suggest that PrsA is a target for proteolysis at the membrane cell wall interface by one of the eight extracytoplasmic proteases missing from the BRB08 strain. This view is supported by the presence of a cell envelope-associated degradation product of PrsA in the parental strain (Figure 6). HtrA and HtrB are probably not involved in PrsA degradation since the levels in the cell envelopes of the BRB08 and BRB14 strains are comparable. Therefore, the elevated levels of PrsA in the medium of the BRB14 strain are probably due to this strain's enhanced propensity for release of cellular proteins into the growth medium.

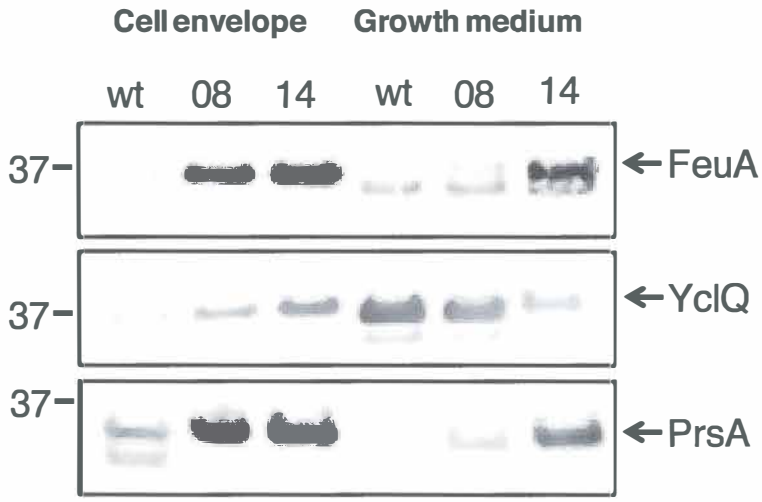

Figure 6. Cell-associated and extracellular forms of the lipoproteins FeuA, YclQ and PrsA. *B. subtilis* strains 168 (wt), BRB08 (08) and BRB14 (14) were cultured as described for Figure 2. Cells were separated from the growth medium by centrifugation, and proteins isolated from the respective samples were analyzed by Western blotting with specific antibodies against FeuA, YclQ or PrsA. Gels were loaded with 2.5 µg of cell envelope proteins and 20 µg of extracellular proteins. The positions of FeuA-, YclQ- or PrsA-specific protein bands are shown. Note that the amounts of cell-associated FeuA and YclQ in this particular experiment appear to be relatively low compared to other independent biological replicates (see chapter 4 of this thesis). The positions of molecular weight markers are indicated on the left.

Impact of protease mutations on membrane proteins

Our analyses identified a total number of 68 membrane proteins in the analyzed growth medium fractions (Figure 5). Remarkably, 24 of these membrane proteins are potential substrates of one or more proteases that are absent from strain BRB08. Six membrane

proteins were exclusively identified in the exoproteome of strain BRB08 (i.e. HtrB, LipA [lipoic acid synthase], YitM, YkuA, YlbC and YvgJ) and eleven in the exoproteome of strain BRB14 (i.e. DacA, GlvA, ManP, MtlA, OxaA1, PtsG, SecDF, SdhA, YhaH, YolF and YtxH). The identification of HtrB in the medium fractions of strain BRB08 was further analyzed by Western blotting with specific antibodies. This revealed that the growth medium of strain BRB08 contained a HtrB-specific protein band with an approximate molecular weight of 37 kDa (Figure 7). In contrast, the cell envelope fraction contained full-size HtrB (~50 kDa) and, as previously reported by Zweers *et al.*, [25] only a minor degradation product of HtrB was detectable in the growth medium of the parental strain 168 (Figure 7).

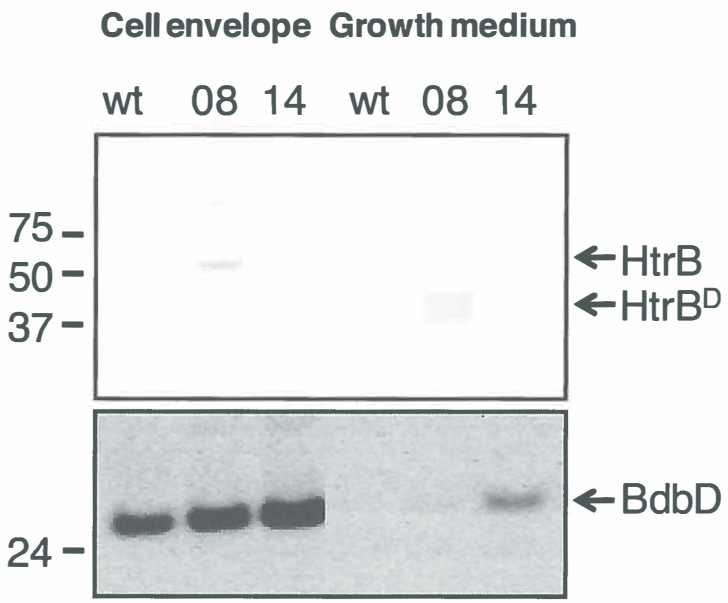

Figure 7. Cell-associated and extracellular forms of the membrane proteins HtrB and BdbD. *B. subtilis* strains 168 (wt), BRB08 (08) and BRB14 (14) were cultured as described for Figure 2, and Western blotting with specific antibodies against HtrB or BdbD was performed as described for Figure 6. The positions of HtrB- or BdbD-specific protein bands are shown. The positions of molecular weight markers are indicated on the left.

Notably, the number of multipass membrane proteins (ManP, MtlA, OxaA1, PtsG and SecDF) was higher in the exoproteome of strain BRB14 than in the exoproteome of strain BRB08. Together with the increased release of cytoplasmic proteins into the growth medium (Figure 4), this is a clear indication for the relatively high instability of the cells of strain BRB14. Thus, it seems that HtrA and HtrB are needed for maintaining cell integrity. Furthermore, the release of multipass membrane proteins by strain BRB14 would suggest that HtrA and HtrB may assist in the regular turnover of such proteins to control their quality. If so, the accumulation of particular malfolded or aggregated membrane proteins in the absence of HtrA and HtrB could lead to disturbance of the membrane integrity and cell lysis. This would be in line with the recent observation of Lamsa *et al.*, [49] who showed that depolarization of the *B. subtilis* membrane resulted in elevated levels of autolysis.

To verify the hypothesis that HtrA and HtrB may be involved in the quality control of regular membrane proteins of

B. subtilis, the crude cell envelope preparations and growth medium fractions of strains 168, BRB08 and BRB14 were analysed by Western blotting for the presence of the membrane protein BdbD. As shown in Figure 7, BdbD was detectable in the growth medium fraction of strain BRB14, but not in those of strains BRB08 and the parental strain 168. This is consistent with the idea that membrane proteins, such as BdbD, are released into the medium due to lysis of cells from strain BRB14. Furthermore, the amounts of BdbD were elevated in the crude cell envelope fractions of strain BRB08 and even more so in the respective fraction of strain BRB14. This confirms the view that HtrA and HtrB are indeed involved in the degradation of native membrane proteins. In fact, this result adds to our previous observation that deletion of the *cssRS* genes resulted in the stabilization of overproduced membrane proteins in *B. subtilis*, which was attributed at least in part to reduced levels of HtrA and HtrB production [25].

Conclusion

In the present studies, we have successfully applied the GeLC-MS approach to expand our knowledge on the roles of extracytoplasmic proteases in the life and death of native extracellular proteins of *B. subtilis* (Figure 8). The most noteworthy observations are (i) that 43 native membrane proteins, lipoproteins and secreted proteins of *B. subtilis* are potential substrates of 8 secreted proteases, (ii) that 14 secreted proteins may require the chaperone function of HtrA and/or HtrB for folding into a protease-resistant conformation, (iii) that HtrA and/or HtrB have a potential shaving function in the

liberation of several lipoproteins from the cell, and (iv) that HtrA and/or HtrB have a potential quality control function in the degradation of membrane proteins. It will be a major challenge for future studies to dissect the individual roles of the different deleted proteases in the observed exoproteome changes, and to determine whether the removal of individual proteases or multiple proteases elicits specific stress responses that impact directly or indirectly on the abundances of the here-identified extracellular proteins of *B. subtilis*. Importantly, we have shown that HtrA and HtrB are needed to maintain cell integrity, which probably relates to the role of these proteins in the quality control of certain membrane-associated proteins. Thus, while previous studies revealed roles of HtrA and HtrB in counteracting the detrimental effects of heat stress or protein secretion stress, our present findings underpin the crucial and very diverse functions of HtrA and HtrB under non-stress conditions.

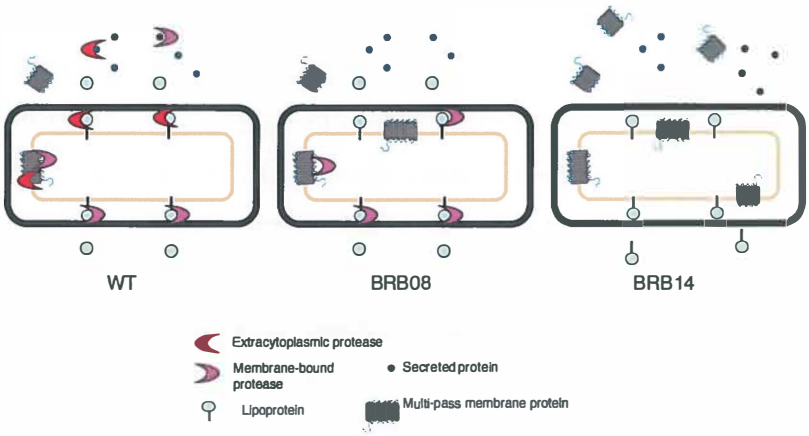

Figure 8. Schematic representation of the different scenarios for extracytoplasmic protein degradation in *B. subtilis* 168 (wt), BRB08 and BRB14.

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Chapter: 3

Degradation of the twin-arginine translocation substrate YwbN by extracytoplasmic proteases of *Bacillus subtilis*

Chapter 3: Degradation of the twin-arginine translocation substrate YwbN by extracytoplasmic proteases of *Bacillus subtilis*

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Abstract

Bacterial twin-arginine translocases can export fully folded proteins from the cytoplasm. Such proteins are usually resistant to proteolysis. Here we show that multiple extracellular proteases degrade the *B. subtilis* Tat substrate YwbN. This suggests that secreted YwbN is either not fully folded, or that folded YwbN exposes protease cleavage sites.

Introduction

Protein folding is a specificity determinant in the transport of proteins across bacterial membranes. Major protein transport machineries, such as the Sec translocase, only translocate unfolded proteins (6). In contrast, the twin-arginine translocase (Tat) is known to translocate fully folded proteins that may even contain cofactors (1, 22, 23, 28, 35). Importantly, the folding state of a protein is also critical for its biological activity and stability. Therefore, proteins have to fold efficiently either before or after membrane translocation, depending on their translocation via the Tat or Sec pathways (4, 9, 16, 27, 28, 30, 31).

Tat translocases are present in Gram-negative and Gram-positive bacteria (1, 5, 13, 22, 29, 31, 35). Proteins are specifically targeted to these Tat translocases by signal peptides that possess a twin-arginine (RR) motif in their N-terminus (1, 8, 11, 22, 23, 31, 35). In *E. coli* it was shown that cofactor-containing Tat substrates require dedicated proofreading-chaperones, like TorD, DmsD, HyaW or NapD for folding and cofactor insertion prior to translocation (8, 23, 25, 29). These chaperones (known as redox enzyme maturation proteins or REMPs) sequester the RR-signal peptides of their substrates until these are properly assembled. Only then the REMPs dissociate from the RR-signal peptide thereby allowing Tat-dependent translocation of the cofactor-containing folded protein (8, 29). This REMP activity has not yet been demonstrated in all bacteria and especially in Gram-positive bacteria, such as *Bacillus subtilis*, the mechanisms for folding and quality control of Tat substrates have remained enigmatic (14,31,34). Notably, proteins

that are not properly folded are rejected by the Tat translocase and degraded. It seems that this quality control process relies on general proteolytic systems that are responsible for the turnover of misfolded proteins (1, 4, 7, 18, 27).

Proteolysis is an important theme in the physiology of *B. subtilis*, which is appreciated both as a model for fundamental scientific research on Gram-positive bacteria, and as a workhorse in the biotechnological production of enzymes and vitamins (9, 31). Proteases produced by *B. subtilis* can be distinguished into 'quality control proteases' and 'feeding proteases' (19, 24, 31). Quality control proteases are generally responsible for degradation of misfolded proteins in the cytoplasm, membrane and cell wall, whereas feeding proteases are secreted to degrade extracellular proteins for the provision of nutrients (3, 9, 24, 31). Extracellular proteases represent major bottlenecks in the production of heterologous proteins that fold inefficiently or expose protease cleavage sites (9,10). Accordingly, it was proposed that it might be beneficial to export heterologous proteins with RR-signal peptides via Tat, thereby allowing them to fold in the cytoplasm prior to export and exposure to the feeding proteases (33). In addition, the deletion of multiple genes for feeding proteases would preclude the degradation of the folded secreted proteins (9, 24, 31).

B. subtilis possesses two Tat translocases called TatAdCd and TatAyCy that operate parallel (12, 14). The TatAdCd translocase is only expressed under conditions of phosphate limitation as is the case for its specific substrate PhoD (13, 26). In contrast, the TatAyCy translocase is constitutively produced (21). To date, the putative Dyp-type peroxidase YwbN is the only protein

known to be specifically secreted via TatAyCy (12). Based on its secretion via Tat, it was generally assumed that YwbN is translocated across the membrane in a folded state (14,31). This would confer resistance to the HtrA and HtrB quality control proteases in the membrane, the wall-bound and secreted quality control protease WprA, as well as the extracellular feeding proteases AprE, Bpr, Epr, Mpr, NprB, NprE and Vpr (9, 30, 31). The present studies were aimed at testing this assumption using a collection of protease mutant strains that lacked increasing numbers of extracellular proteases and quality control proteases (Table 1) all of which are expressed under the tested conditions (21).

Table 1. Strains

Strains	Relevant properties	Reference
B. subtilis		
168	*trpC2*	17
BRB02	*trpC2; ΔnprB, ΔaprE*	Cobra Biologics
BRB03	*trpC2; ΔnprB, ΔaprE, Δepr*	Cobra Biologics
BRB04	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr*	Cobra Biologics
BRB05	*trpC2*; *ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE*	Cobra Biologics
BRB06	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr*	Cobra Biologics
BRB07	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr*	Cobra Biologics

BRB08	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔwprA*	Cobra Biologics
BRB09	*trpC2;ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔhtrA*	Cobra Biologics
BRB10	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr ΔhtrB*	Cobra Biologics
BRB11	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔwprA, ΔhtrA*	Cobra Biologics
BRB12	*trpC2*; *ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔwprA, ΔhtrB*	Cobra Biologics
BRB13	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔhtrA, ΔhtrB*	Cobra Biologics
BRB14	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, ΔwprA, ΔhtrA ΔhtrB*	Cobra Biologics
BRB07 AyCy	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, tatAy-tatCy::Sp*; Sp^R	This study
BRB07 AdCd	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, tatAd-tatCd::Cm;* Cm^R	This study
BRB07 AyCyAdCd	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr, tatAd-tatCd::Cm; tatAy-tatCy::Sp;* Sp^R, Cm^R	This study
BRB07aprE	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr*; pHBaprE; Em^R; Cm^R	This study

BRB07bpr	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr*; pHBbpr; Em^R; Cm^R	This study
BRB07epr	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr*; pHBepr; Em^R; Cm^R	This study
BRB07mpr	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr*; pHBmpr; Em^R; Cm^R	This study
BRB07nprB	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr*; pHBnprB; Em^R; Cm^R	This study
BRB07nprE	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr*; pGS1npr; Cm^R,	This study
BRB07vpr	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE, Δmpr, Δvpr*; pHBvpr; Em^R; Cm^R	This study

BRB = *Bacillus* Recipharm cobra **B**iologics

Results and Discussion

To assess the impact of proteases on the extracellular levels of YwbN, cells were grown in Lysogeny Broth (LB). Next, cells were separated from the growth medium by centrifugation and the amounts of YwbN in both fractions were assessed by Western blotting using specific antibodies. Unexpectedly, compared to the

parental strain 168, a major increase in the levels of extracellular YwbN was observed upon the consecutive deletion of genes for feeding and quality control proteases (Figure 1).

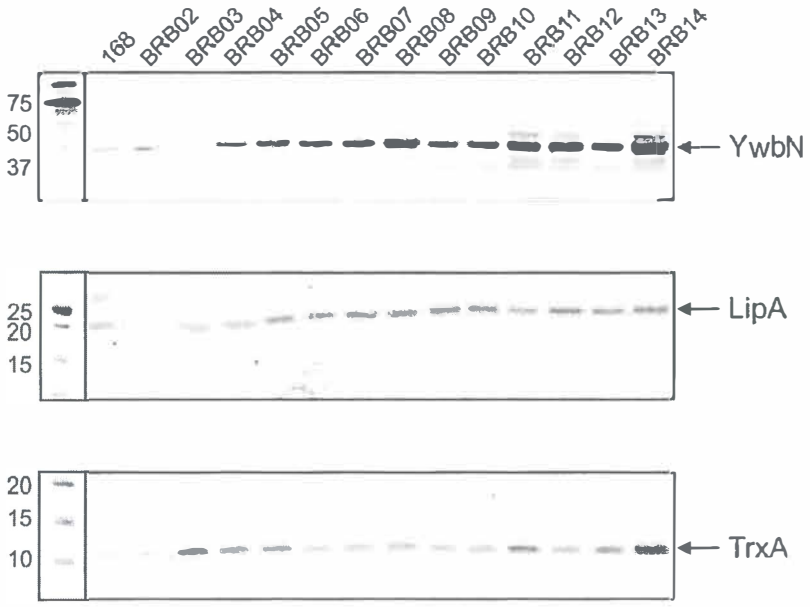

FIGURE 1. Increasing levels of YwbN in the growth media of protease mutants

Cells of *B. subtilis* 168 and BRB02-14 were grown overnight in LB medium at 37°C. The overnight cultures were diluted in fresh LB medium and grown for 6 hours. Cells were then separated from the growth medium by centrifugation. Proteins in the medium fractions were separated using pre-cast 10% Bis-TrisNuPAGE gels (Invitrogen) and semi-dry blotted (75 min at 1 mA/cm2) onto a nitrocellulose membrane. Prior to gel loading all protein samples were corrected for OD_{600}. The presence of YwbN, LipA and TrxA was detected with specific polyclonal antibodies raised in rabbits. Visualization of bound antibodies was performed using IRDye 800 CW goat anti-rabbit secondary antibodies in combination with the Odyssey

Infrared Imaging System (LiCor Biosciences). Fluorescence was recorded at 800 nm. Please note that the apparently reduced LipA level in the medium of strain BRB02 is an outlier that is not normally encountered in this strain. This could relate to an incidental fluctuation in the *lipA* expression level or to a technical problem during sample processing.

In contrast, only a relatively minor change in the levels of the secreted control protein LipA was observed. The increasing levels of YwbN did not relate to cell lysis as evidenced by the extracellular levels of the cytoplasmic protein TrxA, which was previously established as a lysis marker (15). Consistent with this view, the cellular levels of YwbN and TrxA were by-and-large the same in all tested strains (Figure 2).

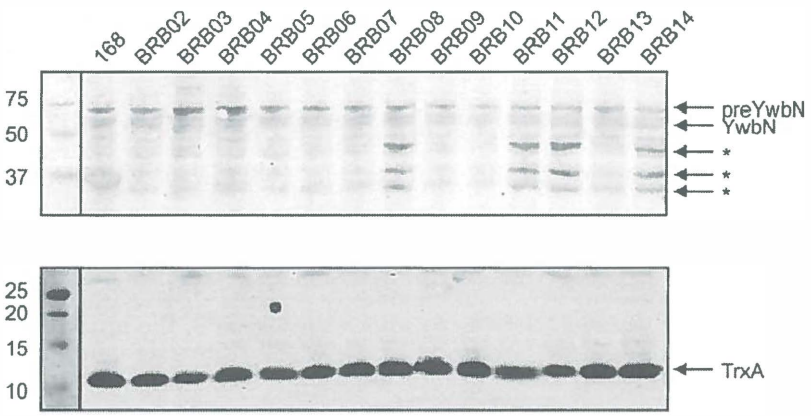

FIGURE 2. Detection of intact and proteolyzed YwbN in protease mutant cells

Cells of *B. subtilis* 168 and BRB02-14 were grown overnight in LB medium at 37°C. The overnight cultures were diluted in fresh LB medium and grown for 6 hours. Cells were collected by centrifugation and

disrupted by bead-beating as previously described (34). Cellular proteins were analyzed by PAGE and Western blotting as described in the legend of Figure 1 using specific antibodies against YwbN and TrxA. Please note that cells lacking the *ywbN* gene give barely any signal with the YwbN-specific antibody in Western blotting experiments as compared to *ywbN* proficient cells (20; data not shown).

Interestingly, all *wprA* mutants contained cell-associated degradation products of YwbN, suggesting that these are normally degraded by the WprA quality control protease. These findings indicated that YwbN is subject to extensive proteolysis during cell wall passage and secretion. To verify this idea and to pinpoint proteases involved in YwbN degradation, a complementation analysis was performed using the seven-fold protease mutant strain BRB07. This mutant can be readily transformed by routine methods (17) in contrast to strains lacking additional proteases (not shown). Briefly, the *nprB*, *aprE*, *epr*, *bpr*, *mpr* or *vpr* genes were PCR-amplified from the genome of *B. subtilis* 168 and cloned into the low-copy expression vector pHB201 (2). The resulting plasmids or the plasmid pGS1npr for *nprE* expression (32) were then used to transform *B. subtilis* BRB07. As shown in Figure 3, the production of AprE, Bpr, NprE or Vpr resulted in decreased extracellular YwbN levels, comparable to that of the parental strain 168. In contrast, ectopic expression of *nprB*, *epr* and *mpr* did not result in relatively low extracellular YwbN levels as encountered in the parental strain 168. Most likely, this means that NprB, Epr and Mpr are not involved in YwbN degradation.

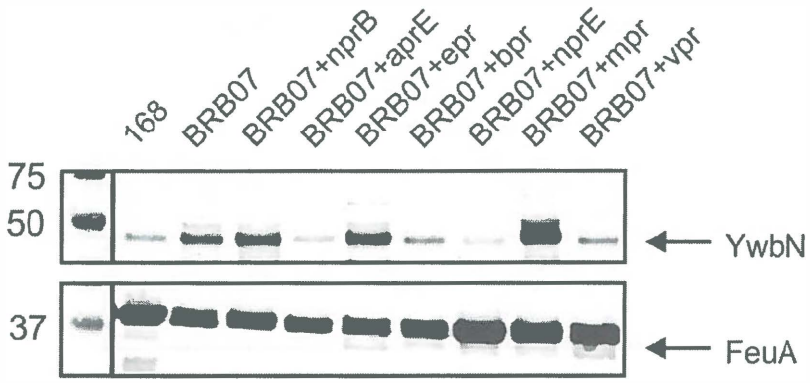

FIGURE 3. Identification of proteases that degrade YwbN

To identify proteases responsible for YwbN degradation, strain BRB07 was transformed with plasmid-borne copies of the proteases genes that had been deleted from this strain. The *nprB*, *aprE*, *epr*, *bpr*, *mpr* or *vpr* genes were cloned in the pHB201 expression plasmid (2). For *nprE* expression the previously constructed plasmid pGS1npr was used (32). The presence of the YwbN and FeuA proteins in growth medium fractions of the transformed strains was detected by PAGE and Western blotting with specific polyclonal rabbit antibodies as described in the legend of Figure 1.

However, we cannot exclude the possibility that, upon ectopic expression of the respective genes, the production levels of NprB, Epr and Mpr are lower than those in the parental strain. In contrast to YwbN, the secreted control protein FeuA was not affected by the ectopic expression of protease genes. Lastly, we showed that YwbN was still TatAyCy-dependently secreted in the BRB07 strain using *tatAyCy*, *tatAdCd* or *tatAdCd-tatAyCy* mutant derivatives (Figure 4).

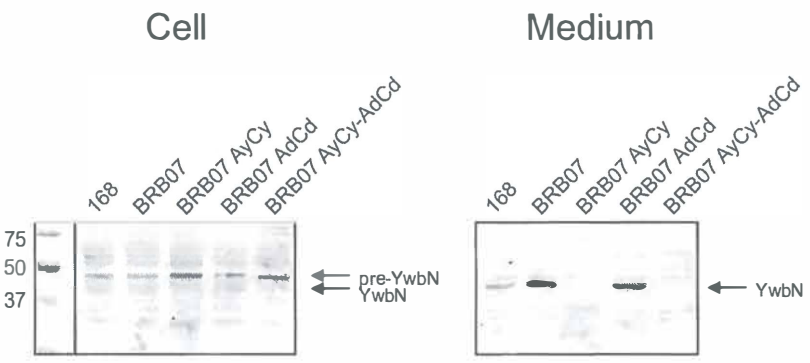

FIGURE 4. Tat-dependent secretion of YwbN in a multiple protease mutant

To verify the Tat-dependent secretion of YwbN by strain BRB07, mutant derivatives of this strain were constructed that lacked the *tatAyCy*, *tatAdCd*, or *tatAyCy-tatAdCd* genes. Next, the levels of YwbN in the growth medium and cells were assayed by Western blotting as described in the legends of Figures 1 and 2.

Taken together, our data show that the Tat-dependently secreted protein YwbN is a substrate for multiple extracellular proteases of *B. subtilis* including both feeding and quality control proteases. Our findings thus challenge the hypothesis that Tat-dependently secreted proteins of *B. subtilis* would be protease-resistant. Instead, our observations suggest that YwbN is either not fully folded upon membrane translocation, cell wall passage and secretion into the growth medium, or that the folded YwbN exposes protease cleavage sites which would be highly remarkable for a native secretory protein of *B. subtilis*. At present, we do not know whether the post-translocational degradation of YwbN is advantageous for *B. subtilis*. Clearly, the degradation of YwbN is not disadvantageous for the

cells under the tested conditions, suggesting that the steady-state production level of this protein is high enough to sustain optimal growth and cell viability.

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Chapter: 4

Degradation of extracytoplasmic catalysts for protein folding in *Bacillus subtilis*

Chapter 4: Degradation of extracytoplasmic catalysts for protein folding in *Bacillus subtilis*

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To be submitted

Abstract

PrsA, HtrA and HtrB of *Bacillus subtilis* are needed for the extracytoplasmic folding and quality control of exported proteins. Here we show that these folding catalysts are substrates of multiple extracytoplasmic proteases. Thus, improved protein secretion by multiple protease mutant strains may relate to reduced proteolysis and elevated chaperone levels.

Introduction

The bacterial Sec pathway for general protein secretion transports proteins in an unfolded state from the cytoplasm to extracytoplasmic locations of the cell, or the extracellular milieu. Accordingly, these proteins need to fold efficiently into their active and protease-resistant conformation once membrane translocation is completed (9, 27, 29, 30, 38). In the Gram-positive bacterium and biotechnological 'workhorse' *Bacillus subtilis*, which is especially appreciated for its high protein secretion capacity, the folding of secreted proteins can be facilitated by at least three different extracytoplasmic chaperones and quality control factors. These are known as PrsA, BdbD and HtrA. PrsA is an essential lipoprotein that is attached to the outer surface of the cytoplasmic membrane through a diacyl-glyceryl modification (17). In *B. subtilis*, PrsA is known to influence the folding of penicillin-binding proteins, such as PBP2a, PBP2b, PBP3 and PBP4 (12, 17). Overexpression of PrsA is known to increase the yields of secreted heterologous proteins, α-amylases in particular (13, 17, 33). The BdbD protein is a thiol-disulphide oxidoreductase that is localized to the outer side of the cytoplasmic membrane by its N-terminal membrane anchor (3, 19). In conjunction with the quinone reductase BdbC, BdbD facilitates the formation of disulphide bonds in competence proteins needed for DNA uptake, such as ComEC and ComGC (8, 10, 20, 23). BdbD also catalyzes oxidative folding of secreted heterologous proteins, such as the alkaline phosphatase PhoA of *Escherichia coli* (3, 5, 18). HtrA is known to combine a chaperone function with a protease function, allowing it to degrade translocated proteins that cannot be productively folded (4). In *B. subtilis* it was shown that

HtrA is needed for folding of the secreted protein YqxI (1). Although this has not been shown experimentally, the HtrA paralogue HtrB is likely to have combined chaperone and protease functions as well (39). Both HtrA and HtrB are bound to the membrane by an N-terminal membrane anchor, but it has to be noted that HtrA is also detectable in the growth medium (1, 24). Under conditions of protein secretion stress, for example due to high-level α-amylase production, the synthesis of HtrA and HtrB is strongly enhanced, which relates to tight regulation by the CssRS two-component regulatory system (6, 11, 24, 25). Furthermore, CssRS mediates the cross-regulation of the *htrA* and *htrB* genes, the expression of one going up if the other one is inactivated (6, 25). Both HtrA and HtrB are needed for cell survival under conditions of severe protein secretion stress (11, 24, 25).

B. subtilis produces at least eight extracellular proteases known as AprE, Bpr, Epr, Mpr, NprB, NprE, Vpr and WprA, which are usually secreted during the stationary growth phase (7, 16, 29). Importantly, the WprA protease is also localised to the cell wall (2, 14, 22, 28). These proteases perform a variety of functions, including the degradation of proteins in organic matter for nutrient provision and the proteolytic processing of other proteins (26, 32). Notably, it was so far believed that, upon correct folding, the native exported proteins of *B. subtilis* would be largely resistant to the extracytoplasmic proteases of this organism (27). On the other hand, it has been known for a long time that the extracellular proteases have a high propensity to degrade heterologous secreted proteins, which often fold slowly upon membrane translocation (34, 35). Though this may ensure product quality control in some cases, it

mostly leads to huge commercial losses. Multiple protease mutant strains of *B. subtilis* were therefore constructed, including the frequently used strains WB600, WB700 and WB800 (1, 36, 37). Indeed, these strains displayed increased levels of heterologous protein secretion, which was attributed to reduced proteolysis. However, to date it was never assessed whether the absence of extracytoplasmic proteases might also have a positive impact on the levels of extracytoplasmic chaperones and quality control factors, which could be beneficial for the quality and yields of secreted heterologous proteins. The present studies were therefore aimed at determining the impact of extracytoplasmic proteases on the levels of PrsA, BdbD, HtrA and HtrB. For this purpose a series of strains with multiple markerless protease gene deletions were used (Table 1).

Table 1. Strains

Strains	Relevant properties	Reference
B. subtilis		
168	*trpC2*	(21)
BRB02	*trpC2; ΔnprB, ΔaprE*	Cobra Biologics
BRB03	*trpC2; ΔnprB, ΔaprE, Δepr*	Cobra Biologics
BRB04	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr*	Cobra Biologics
BRB05	*trpC2*; *ΔnprB, ΔaprE, Δepr, Δbpr, ΔnprE*	Cobra Biologics
BRB06	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr,*	Cobra

	ΔnprE, Δmpr	Biologics
BRB07	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr,*	Cobra
	ΔnprE, Δmpr, Δvpr	Biologics
BRB08	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr,*	Cobra
	ΔnprE, Δmpr, Δvpr, ΔwprA	Biologics
BRB09	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr,*	Cobra
	ΔnprE, Δmpr, Δvpr, ΔhtrA	Biologics
BRB10	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr,*	Cobra
	ΔnprE, Δmpr, Δvpr ΔhtrB	Biologics
BRB11	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr,*	Cobra
	ΔnprE, Δmpr, Δvpr, ΔwprA, ΔhtrA	Biologics
BRB12	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr,*	Cobra
	ΔnprE, Δmpr, Δvpr, ΔwprA, ΔhtrB	Biologics
BRB13	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr,*	Cobra
	ΔnprE, Δmpr, Δvpr, ΔhtrA, ΔhtrB	Biologics
BRB14	*trpC2; ΔnprB, ΔaprE, Δepr, Δbpr,*	Cobra
	ΔnprE, Δmpr, Δvpr, ΔwprA, ΔhtrA	Biologics
	ΔhtrB	
ΔwprA	*trpC2; wprA*::Cm^R	(15)
BRB	***Bacillus*** **R**ecipharm cobra **B**iologics	

Results and Discussion

Upon growth of different protease mutant strains in Lysogeny Broth (LB), the cells were separated from the growth medium by centrifugation. To assess the PrsA levels, both fractions were analyzed by Western blotting using PrsA-specific antibodies. As

controls, the levels of the lipoproteins FeuA and YclQ were monitored with specific antibodies. This revealed a cell-associated degradation product of PrsA (denoted $PrsA^D$), which was absent from the multiple protease mutant strains BRB08, 11, 12 and 14 (Figure 1).

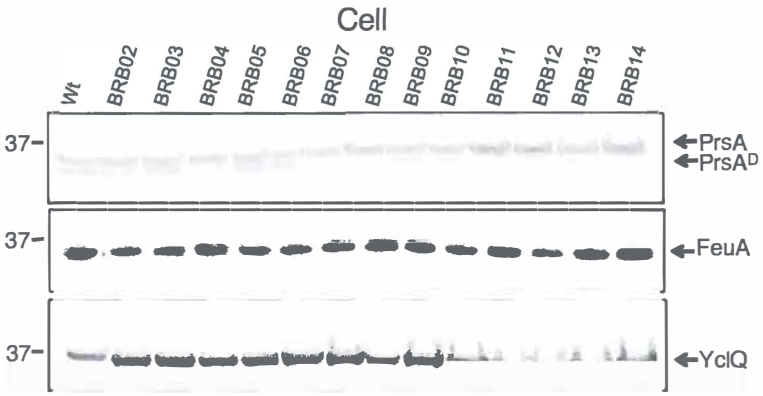

FIGURE 1. The cell-associated lipoprotein PrsA is a potential WprA substrate. Cells of *B. subtilis* 168 (wt) and the protease mutants BRB02-14 were grown overnight in LB medium at 37°C. The overnight cultures were diluted in fresh LB medium and growth was continued until 2 h after entry into the stationary phase. Cells were then separated from the growth medium by centrifugation and disrupted by bead-beating as previously described (31). Cellular proteins were separated on pre-cast 10% Bis-TrisNuPAGE gels (Invitrogen) and semi-dry blotted (75 min at 1 mA/cm2) onto a nitrocellulose membrane. Prior to gel loading all protein samples were corrected for OD_{600}. Specific polyclonal antibodies raised in rabbits were used to detect the blotted PrsA, FeuA and YclQ proteins. Bound antibodies were visualized using IRDye 800CW goat anti-rabbit secondary antibodies in combination with the Odyssey Infrared Imaging System (LiCor Biosciences). Fluorescence was recorded at 800 nm. The positions

of intact PrsA, a degradation product of PrsA ($PrsA^D$), intact FeuA and intact YclQ are marked with arrows. Please note that the absolute amounts of PrsA in WprA-deficient cells were found to vary in different independent biological replicates, but they were always significantly higher than in WprA proficient cells (chapter 2 of this thesis). The position of a 37 kDa molecular weight marker is indicated.

These four strains lack the WprA protease, which indicated that the cell wall-bound form of WprA is involved in PrsA cleavage. In contrast, the lipoproteins FeuA and YclQ were apparently not degraded in cells of the tested strains. Subsequent inspection of the respective growth medium fractions revealed that all protease mutants lacking WprA released massive amounts of PrsA into the growth medium (Figure 2). This implies that WprA has a major role in the 'shaving' and/or the degradation of PrsA, A possible role of WprA in PrsA shaving would suffice to explain the release of PrsA into the medium.

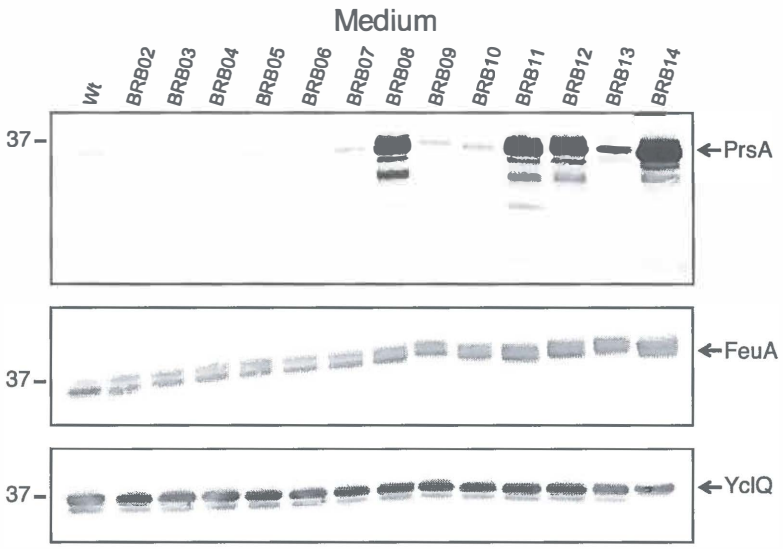

FIGURE 2. Multiple protease mutants lacking WprA accumulate PrsA in the growth medium. The presence of PrsA, FeuA and YclQ in growth medium fractions of *B. subtilis* 168 (wt) and the protease mutants BRB02-14 was analyzed by Western blotting as described in the legend of Figure 1. The positions of PrsA-, FeuA- and YclQ-specific bands are marked with arrows. Note that only the major PrsA band is marked, but that smaller PrsA degradation products are also detected. The position of a 37 kDa molecular weight marker is indicated.

Notably, the dominant released form of PrsA in the medium of multiple protease mutant strains lacking *wprA* was not detectable in the medium of single *wprA* mutant cells (Figure 3).

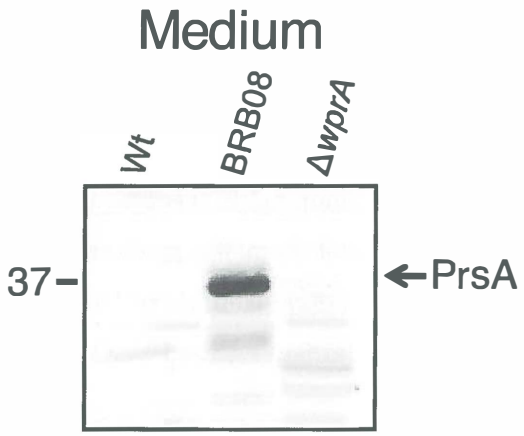

FIGURE 3. Extracellular degradation of PrsA in a *wprA* single mutant strain. The presence of PrsA in the growth medium fractions of *B. subtilis* 168 (wt), BRB08 and a *wprA* single mutant strain was analyzed by Western blotting with antibodies against PrsA as described in the legend of Figure 1. The position of the major PrsA-specific band in the medium of strain BRB08 is marked with an arrow; smaller degradation products of PrsA are detected but not marked. The position of a 37 kDa molecular weight marker is indicated.

Instead, smaller degradation products of PrsA were detectable in the medium of the single *wprA* mutant, showing that one or more of the seven proteases lacking from strain BRB07 are responsible for the degradation of PrsA that is liberated from the cells when WprA is present. Furthermore, it is noteworthy that the absence of HtrA and HtrB from strains BRB13 and 14 adds to the amount of extracellular PrsA (Figure 2), indicating that these proteases are also involved in the liberation of PrsA from the cells. Nevertheless, the absence of HtrA and/or HtrB does not result in elevated levels of cell-associated PrsA (Figure 1). In the case of FeuA, we observed two

released forms, the lower Mw form being dominant in the medium of the parental strain 168 and the higher Mw form being dominant in the medium of strain BRB14 that lacks the largest number of extracytoplasmic proteases (Figure 2). Similarly, two forms of YclQ were released into the medium, but in this case the lower Mw form was even completely absent from the medium of strain BRB14 (Figure 2). This indicates a dominant role of HtrA and HtrB in the generation of the low Mw form of released YclQ.

In contrast to PrsA, multiple protease gene deletions had no detectable effects on the cellular BdbD levels (Figure 4). However, substantially increased amounts of full-size BdbD were detectable in the medium fractions of strains BRB11 and 14, both of

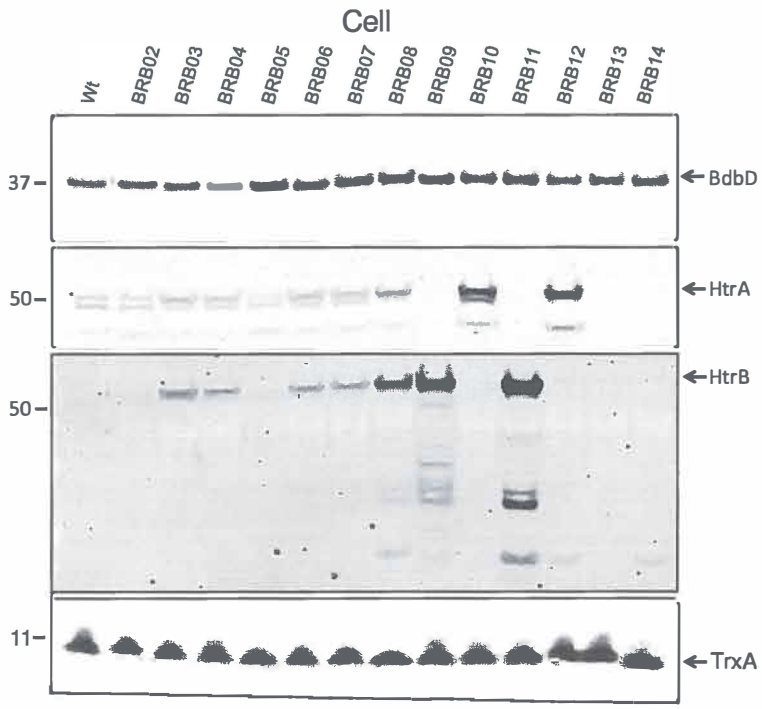

FIGURE 4. Levels of BdbD, HtrA and HtrB in cells of multiple protease mutant strains. The presence of BdbD, HtrA, HtrB and the cytoplasmic marker protein thioredoxin A (TrxA) in cell fractions of *B. subtilis* 168 (wt) and the protease mutants BRB02-14 was analyzed by Western blotting as described in the legend of Figure 1. The positions of BdbD-, HtrA-, HtrB- and TrxA-specific bands are marked with arrows. Note that only the major HtrA and HtrB bands are marked, but that smaller HtrA and HtrB degradation products are also detected. The positions of 37 kDa, 50 kDa and 11 kDa molecular weight markers are indicated.

which lack WprA *plus* HtrA. Since the media of these strains also contain elevated levels of the cytoplasmic marker protein TrxA (Figure 5), the most likely explanation of this finding is that the full-size BdbD was released into the medium due to elevated levels of cell lysis. However, reduced levels of BdbD degradation due to the absence of WprA and HtrA cannot be excluded completely since the levels of extracellular TrxA were also increased in strains BRB12 and 13, which show much less extracellular BdbD (Figure 5).

Lastly, the levels of HtrA and HtrB in the cells and growth media of multiple protease mutant strains were compared (Figures 4 and 5). Intriguingly, also these two proteins appear to be substrates of WprA since the cellular levels of both HtrA and HtrB were elevated in the multiple protease mutant BRB08 lacking the *wprA* gene. Additionally, highly elevated levels for HtrA were detected in *htrB* mutant cells, and highly elevated levels of HtrB were detected in *htrA* mutant cells. This is most likely due to the CssRS-dependent

cross-regulation of *htrA* and *htrB* (6, 25). The deletion of only two extracytoplasmic proteases (NprB and AprE) in the BRB02 mutant caused already an elevated level of HtrA in the growth medium, which is in line with the previous observation that the extracellular HtrA levels are increased in multiple protease mutant cells (1, 34, 36). The increased level of extracellular HtrA in the BRB02 mutant does not relate to lysis as evidenced by blotting for the cytoplasmic marker protein TrxA (Figure 5). In contrast to HtrA, an almost linear increase in the accmulation of HtrB in the growth medium was observed when increasing numbers of protease genes were deleted (Figure 5). This cumulative effect of multiple protease mutations shows that HtrB is more sensitive to extracytoplasmic degradation than HtrA. Notably, the highest levels of HtrA and HtrB in the growth medium fractions were observed in strains lacking either *htrA* or *htrB*, which most likely reflects the afore-mentioned cross-regulation between these two genes (6, 25).

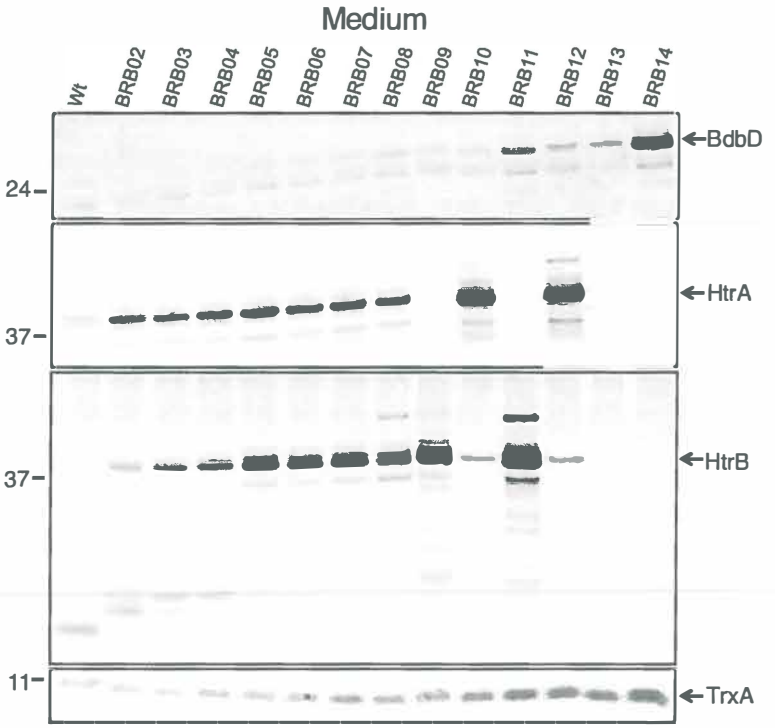

FIGURE 5. Accumulation of full-size BdbD and degradation products of HtrA and HtrB in the growth media of multiple protease mutant strains. The presence of BdbD, HtrA, HtrB and the cytoplasmic lysis marker TrxA in growth medium fractions of *B. subtilis* 168 (wt) and the protease mutants BRB02-14 was analyzed by Western blotting as described in the legend of Figure 1. The positions of BdbD-, HtrA-, HtrB- and TrxA-specific bands are marked with arrows. Note that only the major HtrA and HtrB bands are marked, but that smaller HtrA and HtrB degradation products are also detected. Furthermore, the anti-HtrB antibodies display some minor cross-reactivity with the major extracellular form of HtrA. The positions of 24 kDa, 37 kDa and 11 kDa molecular weight markers are indicated.

Taken together, our present findings show that the folding catalyst PrsA is targeted by WprA which, together with HtrA and HtrB, seems involved in PrsA release into the medium. The released PrsA is then subject to degradation by other extracellular proteases. Furthermore, HtrA and HtrB are prone to proteolysis by cell-associated WprA and proteases in the medium. Together, these findings suggest that the elevated levels of secreted (heterologous) proteins in multiple protease mutant strains may not only be due to reduced degradation, but also to elevated levels of cell-associated protein folding catalysts, in particular PrsA, and quality control factors like HtrA and HtrB.

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Chapter: 5

Summary, general discussion and perspectives

Samenvatting, algemene discussie en perspectief

Chapter 5: Summary, general discussion and perspectives

Bacillus subtilis is a Gram-positive rod-shaped bacterium inhabiting the top layers of the soil and the plant rhizosphere. This bacterium is continuously challenged by the ever-changing conditions in its environment. Accordingly, it has developed a wide array of strategies and mechanisms to avoid or overcome adverse situations. For example, *B. subtilis* produces antibiotics, such as sublancin 168, to compete with other organism for available nutrients that may be present in limiting quantities [1, 2]. Alternatively, flagellum synthesis empowers *B. subtilis* cells to avoid competition by migrating to more favorable environments [3, 4]. The uptake of K^+ and increased synthesis of proline and glycine betaine is an effective strategy to survive under high osmolarity conditions [5-9], which exemplifies nicely how physiological adaptations allow *B. subtilis* to overcome severe environmental insults. Development of competence for the uptake of foreign genetic material is a more complex adaptive strategy that potentially increases the robustness of a *Bacillus* population by generating genetic diversity [10]. Finally, if such adaptations do not suffice for cell survival, *B. subtilis* can overcome extremely harsh conditions in the form of spores until the environment becomes more favorable again [11, 12].

The early availability of the complete genome sequence of *B. subtilis* has made it an ideal candidate among the Gram-positive bacteria for fundamental research [13, 14]. Furthermore, the inherent nature of *B. subtilis* to secrete large quantities of native proteins also makes it an ideal model organism for application-oriented studies on the secreted proteins and the protein secretory machinery. Such studies have indeed been extremely valuable for progress in biotechnological applications of *B. subtilis*, especially where it came to the secretion of proteins with high commercial value [15-19]. The status of *B. subtilis* as a Generally Recognized As Safe (GRAS) microorganism has further stimulated its exploitation as an ideal work-horse for use in the

biotechnology industry for the production of high-value secreted proteins and vitamins.

A concise overview of the 'secretome' of *B. subtilis* is presented in Chapter 1 of this thesis. By definition, the secretome includes both the protein secretion machinery and the secreted proteins of an organism [20]. Chapter 1 also addresses possibilities for secretome engineering in *B. subtilis*, which is needed to meet the ever-increasing demands for enzymes and other proteins of biomedical and industrial relevance. Previous efforts in this area have been focused on signal peptides that target particular proteins to membrane-embedded protein translocation machineries, like the Sec and Tat translocases, as well the Sec and Tat secretion pathways themselves. Such efforts were, for example, focused on optimizing extracytoplasmic folding catalysts like the peptidyl-prolyl cis/trans isomerase PrsA, or the thiol-disulfide oxidoreductase BdbD. While enhanced post-translocational protein folding is one possible approach to increase product yields, an alternative and more pragmatic approach involves the reduction of protein degradation. This has focused strong interest on *Bacillus* proteases residing at the membrane-cell wall interface, in the cell wall, and in the exoproteome [21]. Such proteases include the membrane-bound HtrA and HtrB proteases, the wall protease WprA, and the secreted proteases AprB, AprE, Bpr, Epr, NprB, NprE, and Vpr. Notably some of these proteases, like HtrA and WprA, have dual localizations as their processed forms are also encountered in the exoproteome. The known functions of these ten different enzymes are diverse, but they do overlap to some extent. Briefly, the HtrA and HtrB proteases have important roles in protein quality control, the WprA protease is involved in protein quality control and degradation, and the secreted proteases have functions in the provision of nutrients as well as the processing of other secreted proteins. These functions have been discussed in

more detail in **Chapter 1**, thereby setting the stage for the research described in this thesis.

The secreted proteases of *B. subtilis* are predominantly expressed during the stationary growth phase when, for example, certain nutrients are available in limited amounts. In the natural habitats of *B. subtilis*, the secreted extracellular proteases AprE, Bpr, Epr, Mpr, NprB, NprE, VprA and WprA have thus been implicated in the degradation of organic matter or proteinaceous material to simpler molecules like peptides and amino acids, which then serve as nutrients for growth [22-32]. Possibly, these proteases degrade also potentially toxic proteins and peptides thereby serving in the protection of the *Bacillus* cell. Roles of extracellular proteases in the processing of other exported proteins, such as the major wall protein WapA, and autolysins like LytE and LytF have also been reported [33, 34]. Extracellular proteases are furthermore known to impact on swarming motility, the processing of lantibiotics and processing of the Phr pentapeptide proCSF [35-37].

The membrane-bound proteases HtrA and HtrB belong to the HtrA family of serine proteases that also have chaperone-like activities [38]. The expression of *htrA* and *htrB* during situations of 'secretion stress' caused by the overproduction of various secretory proteins is governed by the two-component regulatory system CssRS. HtrA of *B. subtilis* is known to possess both protease and chaperone functions, and this is probably also true for HtrB [39, 40]. Based on proteomics and molecular biological analyses, it has been proposed that the protein of unknown function YqxI of *B. subtilis* is a potential substrate for the chaperone activity of HtrA [41]. In pathogenic Gram-positive bacteria, such as *Staphylococcus aureus*, HtrA is known to be very important for pathogenesis, suggesting critical roles in the quality control of fitness- and virulence factors [42].

Extracellular proteases often degrade secreted heterologous proteins thereby causing commercial loss in their biotechnological production. The use of multiple protease-deficient strains, such as the strains WB600, WB700 and WB800 has helped to improve the yields of such vulnerable proteins, at least in laboratory settings [43-45]. The overproduction of HtrA and HtrB under conditions of secretion stress will also result in the degradation of secreted heterologous proteins and, in fact, also overproduced membrane proteins seem to be targets of these membrane-bound proteases. Altogether, the secreted proteases and the membrane-bound proteases degrade slowly folding proteins or proteins that expose protease cleavage sites. Notably, slow folding and exposure of cleavage sites is more likely to occur for heterologously produced proteins and, accordingly, extracytoplasmic proteases represent serious bottlenecks in the secretion of heterologous proteins [39, 46, 47].

The research described in this thesis focuses on the roles of the eight extracellular proteases AprE, Bpr, Epr, Mpr, NprB, NprE, VprA and WprA, and the two membrane-bound proteases HtrA and HtrB in the degradation of a range of other native exported proteins of *B. subtilis*. This was necessary since, to date, very little was known about the stability and ultimate fates of exported *Bacillus* proteins at the proteome-wide level, especially under non-stress conditions. Therefore, the first experimental chapter in this thesis involved an unbiased hypothesis-driving proteomics analysis of the exoproteomes of multiple protease mutant strains (Chapter 2). Subsequent studies described in the two other experimental Chapters 3 and 4 addressed the roles of extracytoplasmic proteases in the degradation of particular substrates. Notably, a completely novel set of strains was used for this purpose that has multiple marker-less gene deletions for up to ten extracytoplasmic proteases. These strains, denoted as *B. subtilis* BRB02-14, were most generously provided by Dr. Rocky Cranenburgh and Prof. Colin Harwood prior to publication.

A previous two-dimensional (2D) gel-based proteomics analysis of *B. subtilis* strains deficient for certain extracellular proteases had clearly indicated the role of extracellular proteases, especially Epr, in the turnover of the native cell wall-binding protein WapA [34]. While 2D-PAGE has been of key importance in the initial exploration of the *Bacillus* secretome, the 2D-gel-based proteomics approaches do not identify proteins with extremely high or low pI's, hydrophobic proteins, as well as proteins with a molecular weight above 250kDa and below 10kDa. Some of these limitations can be overcome by combing the separation of proteins through one-dimensional PAGE with liquid chromatography (LC) and mass spectrometry (MS) in the so-called GeLC-MS approach [48]. **Chapter 2** describes the application of GeLC-MS for a detailed analysis of the exoproteomes of strain BRB08, lacking the eight extracytoplasmic proteases AprE, Bpr, Epr, Mpr NprB, NprE, Vpr and WprA, and strain BRB14, lacking these eight extracytoplasmic protease *plus* the two membrane-bound proteases HtrA and HtrB. An in-depth comparison of the exoprotemes of these two protease-mutant strains and their parental strain 168 resulted in the identification of many potential substrates of the extracellular and membrane-bound proteases of *B. subtilis*. These proteolyzed proteins comprise native secreted proteins, lipoproteins and membrane proteins. The results as summarized in Figure 1 can be interpreted as follows:

- 138 identified proteins in the exoproteome of *B. subtilis* 168 were not detectably affected by the presence or absence of the ten extracytoplasmic proteases that are absent from strain BRB14. Here it should, however, be borne in mind that the results of the applied GeLC-MS approach are qualitative rather than quantitative. Thus, different extracytoplasmic proteins may actually influence the quantities of these 138 proteins, as evidenced for a few lipoproteins for which specific antibodies were available (see Chapters 2 and 4).

- 18 identified proteins were only detectable in the exoproteome of strain 168. This indicates that their presence in the exoproteome depends on activity of one or more proteases that are absent from strains BRB08 and 14. Conceivably, this could involve WprA, which has been implicated in protein quality control.
- 15 identified proteins were only detectable in the exoproteome of strains 168 and BRB08. This suggests that these proteins may require HtrA and/or HtrB for folding into a stable conformation and/or for their release into the growth medium. As nine of these proteins are lipoproteins, a role of HtrA and HtrB in the general phenomenon of 'lipoprotein shaving' seems likely (Figure 2). However, lipoprotein shaving is not completely blocked in strain BRB14, so other membrane proteases that were not investigated in the present analyses could also be involved in this process.
- 22 identified proteins were only detectable in the exoproteome of strain BRB08. This suggests that these proteins are substrates of one or more of the eight proteases lacking from strain BRB08 and that they require HtrA and/or HtrB for folding into a stable conformation.
- 262 identified proteins were only detectable in the exoproteomes of strains BRB08 and 14. This might suggest that these proteins are substrates of one or more of the eight proteases lacking from strain BRB08. However, strains BRB08 and 14 show increased lysis, as evidence by the increased extracellular TrxA levels. Therefore, especially the 219 cytoplasmic proteins in this category may reflect increased cell lysis rather than reduced proteolysis, or a combination of these two effects.
- 227 identified proteins were only detectable in the exoproteome of strain BRB14. It seems highly unlikely that all of these proteins are

substrates of HtrA and/or HtrB. Instead, the significantly increased lysis of strain BRB14 (compared to strains 168 and BRB08) seems to lead to a massive release of cytoplasmic proteins into the growth medium. Therefore, it seems more reasonable to regard only the typical membrane proteins (11), lipoproteins (2) and secreted proteins (1) in this category as potential substrates of HtrA and/or HtrB. However, also these membrane proteins and lipoproteins may be released due to cell lysis, as seems to be the case for the membrane protein BdbD (Figure 2).

- 4 identified proteins were only detectable in the exoproteomes of strains 168 and BRB14. Following the reasoning applied above for the interpretation of the proteomics data, these four proteins would require a protease that is absent from strain BRB08 and present in strain BRB14. Obviously, this does not seem to make much sense, and it is much more likely that these four proteins reflect the degree of biological variation in the present proteomics analyses.

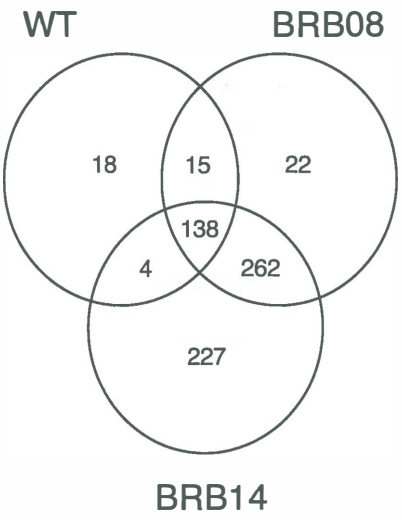


Figure 1. Comparison of proteins identified in the exoproteomes of the multiple protease mutant *B. subtilis* strains BRB08 and 14, and their parental strain 168. Proteins in exoproteome samples of the *B. subtilis* protease mutants BRB08 (08) and BRB14 (14) or the parental strain 168 (wt) were identified by GeLC-MS analyses (Chapter 2). The Venn diagram shows the numbers of proteins that are either unique for each strain or common for two or three strains.

It is important to note here that the lack of detection of a particular protein in a proteomics analysis does not necessarily mean that this protein is not present in the analyzed sample. Such a protein may simply have escaped detection for technical reasons, or due to a certain degree of 'noise' caused by biological fluctuations. Most likely, this applies to the four proteins that were only detected in the media of strains 168 and BRB14. Extrapolated to the entire dataset, this would suggest that only a limited number of 'absent proteins' in the different samples are absent due to technical limitations or biological noise. This is an important conclusion, which increases the confidence in our interpretation of the roles of extracytoplasmic proteases in exoproteome biogenesis. In this context, it is furthermore relevant to note that the impact of multiple protease mutations on the expression of particular genes has not yet been investigated. Hence, some of the differences observed at the exoproteome level may in fact reflect gene regulatory responses. This is an aspect of the present PhD thesis research that needs to be addressed in future studies.

Another issue concerns the best way of preparing samples for proteomics and Western blotting analyses. In Chapter 2 of this thesis, equal amounts of proteins were loaded onto all gels. While the use of equal amounts of proteins in different samples is advantageous for gel-free proteomics analyses, this approach has disadvantages for gel-based methods, especially when different samples display larger differences as was the case for samples from the parental *B. subtilis* strain 168 and its multiple protease-deficient derivatives.

Retrospectively, sample loading with a correction for the optical density of the different cultures, as was done in Chapters 3 and 4, is probably a better option, especially for Western blotting analyses.

Based on the very high susceptibility of strain BRB14 to cell lysis, we hypothesize that the absence of both HtrA and HtrB could lead to the impaired turnover of certain membrane proteins. In turn, this would lead to a destabilization and ultimately disruption of membrane integrity, which would then result in a release of cytoplasmic proteins into the growth medium. Altogether, the exoproteome analyses of *B. subtilis* strains lacking multiple extracytoplasmic proteases give first valuable clues as to which proteins are targeted by these proteases, and the results are also indicative of possible regulatory and quality control mechanisms that determine the composition of the extracellular proteome. To further validate our conclusions from the exoproteome analyses with multiple protease mutant strains of *B. subtilis*, the roles of extracytoplasmic proteases were assessed in more detail in the research that is described in Chapters 3 and 4 of this thesis.

Chapter 3 documents studies on the role of extracytoplasmic proteases in the degradation of the YwbN protein of *B. subtilis*. Together with the phosphodiesterase PhoD, the Dyp- type peroxidase YwbN is currently the best-studied protein that is exported via the *Bacillus* Tat pathway. Specifically, YwbN is secreted via the TatAyCy translocase [49, 50]. As the Tat pathway is known to have a preference for translocation of fully folded proteins, one would expect that extracytoplasmic proteases can do little harm to Tat-dependently secreted proteins, such as YwbN. This idea is based on the fact that fully folded proteins are generally much less susceptible to proteolysis than unfolded proteins. Accordingly, it had been hypothesized that the secretion of proteins in a folded state via the Tat pathway would render them less susceptible to degradation by the extracytoplasmic proteases of *B. subtilis* [51].

This idea was tested with the help of the multiple protease-deficient strains BRB02-BRB14. Unexpectedly, an almost linear increase in the levels of extracellular YwbN was observed, showing that this Tat-dependently secreted protein is subject to degradation by multiple extracellular proteases (Figure 2). Indeed, complementation studies pinpointed AprE, Bpr, NprE and Vpr as being involved in the degradation of YwbN. Furthermore, cellular fractions of protease-deficient strains lacking WprA showed a specific degradation pattern of YwbN, suggesting that also WprA is involved in the degradation of YwbN (Figure 2). These findings show that the extracellular level of YwbN is determined through proteolysis, which is indicative of a quality control function. This view is supported by recent unpublished data from Monteferrante *et al.*, which show that WprA can interact directly with the TatAyCy translocation channel and has a role in the biogenesis of YwbN (personal communication Carmine Monteferrante). Taken together, the results presented in Chapter 3 challenge the dogma that fully folded proteins secreted via the Tat pathway would be more protease resistant than proteins secreted in an unfolded state via the Sec pathway. Several explanations for the observed degradation of YwbN are conceivable, including the secretion of YwbN in a partially unfolded state, the exposure of protease cleavage sites in secreted YwbN, and the regulated turnover of YwbN in accordance with the need for its enzymatic activity.

In **Chapter 4** the effects of extracellular proteases on the extracytoplasmic folding catalysts PrsA and BdbD, and the quality control factors HtrA and HtrB are presented. The lipoprotein PrsA acts as a protein folding catalyst for several penicillin-binding proteins (PBP2a, PBP2b, PBP3, and PBP4) [52]. Various studies have shown that the overproduction of PrsA significantly increases the secretion of α-amylases, the protective antigen of *Bacillus anthracis*, and interferon-β (h-IFN β)[17, 53, 54].

Interestingly, the analysis of PrsA in cells of the multiple protease-deficient strains revealed that less degradation of cell-associated PrsA occurred in strains lacking WprA (Figure 2). Furthermore, high levels of PrsA were detectable in the media of these *wprA* mutant strains, suggesting that the released PrsA is normally degraded by one or more extracellular proteases (Figure 2). This suggests that the increased secretion of particular proteins by multiple protease-mutant *Bacillus* strains that lack WprA may be due both to elevated levels of PrsA and reduced levels of proteolysis. It should be noted that deletion of only the *wprA* gene did not result in enhanced cellular or extracellular PrsA levels, indicating that any beneficial effect of the absence of WprA requires also the absence of other extracytoplasmic proteases. In contrast to PrsA, the cellular levels of the thiol oxidase BdbD, which is needed for disulfide bond formation in extracytoplasmic proteins of *B. subtilis* [55, 56], were not affected by multiple protease mutations. Nevertheless, the media of strains BRB11 and BRB14 contained increased levels of BdbD. As these two strains showed increased levels of lysis, this finding suggests that cell lysis is an important parameter in the release of BdbD from the cells. On the other hand, reduced levels of BdbD degradation due to the absence of WprA and HtrA cannot be excluded completely (Figure 2), because the levels of extracellular TrxA were also increased in strains BRB12 and 13, which show much less extracellular BdbD.Strongly elevated levels of HtrA or HtrB were observed in the cellular and growth medium fractions of strains lacking either *htrA* or *htrB*. This probably relates to the CssRS-dependent cross-regulation of *htrA* and *htrB*. Similar to what was observed for PrsA, the levels of HtrA and HtrB were significantly increased in cells of multiple protease mutant strains lacking WprA, which implicates WprA in the degradation of HtrA and HtrB (Figure 2). Consistent with previously published findings, an increased level of HtrA was

observed in the growth medium of strains BRB02-08, suggesting that in particular AprE and NprB are responsible for extracellular degradation of HtrA. Interestingly, an almost linear increase in the extracellular accumulation of HtrB was detected in the protease mutants BRB02-BRB08. This observation indicates that HtrB is more prone to proteolysis than HtrA, and that multiple extracellular proteases are involved in HtrB degradation. Thus, it seems that PrsA, HtrA and HtrB, upon liberation from the cell membrane by WprA, are subjected to proteolysis by one or more extracellular proteases of *B. subtilis*. These findings show that the cell wall-bound and secreted protease WprA has an even greater impact on the integrity and quality of exported proteins than was previously believed [57-60]. Importantly, the increased cellular levels of HtrA and HtrB in multiple protease mutant cells lacking WprA have a high potential to contribute to improved protein quality control at the membrane-cell wall interface, where proteins emerge from the Sec channel and need to fold rapidly in order to minimize their proteolysis.

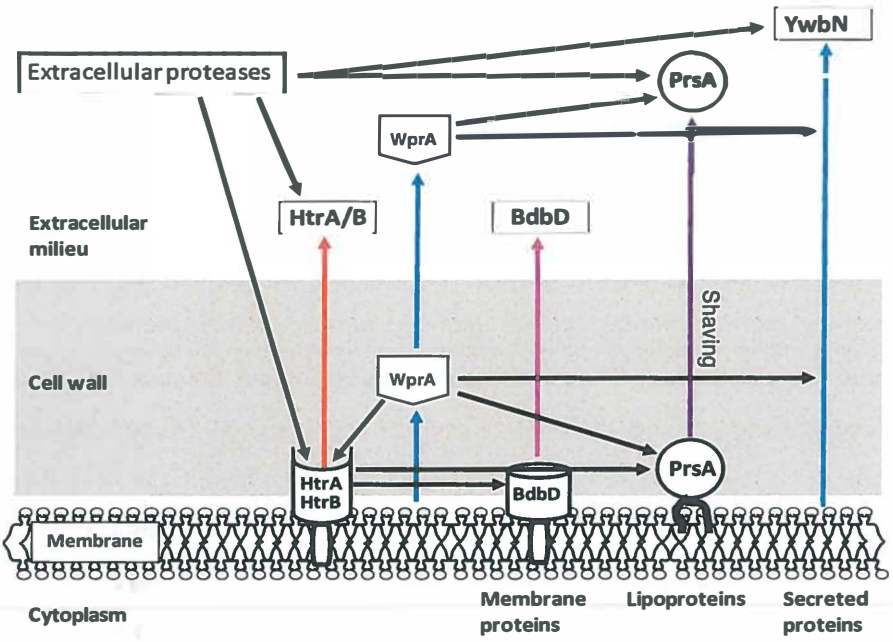


Figure 2. Schematic representation of extracytoplasmic proteases and their potential targets in *B. subtilis*. *B. subtilis* produces at least eight extracellular proteases named AprE, Bpr, Epr, Mpr, NprB, NprE, Vpr and WprA. The WprA protease is also active at the membrane-cell wall interface. In addition, *B. subtilis* has at least two membrane-associated proteases named HtrA and HtrB. The extracellular and membrane-associated proteases are collectively termed extracytoplasmic proteases. Potential targets of extracytoplasmic proteases were identified by proteomics and Western blotting analyses (marked by black arrows). HtrA and HtrB are targeted for proteolysis by the extracellular proteases and WprA, leading to the release of these proteins from the membrane (orange arrow). HtrA or HtrB are involved in the release and degradation of the membrane protein BdbD (pink arrow). WprA and HtrA or HtrB are involved in the shaving of the lipoprotein PrsA (purple arrow). In addition, the shaved PrsA is targeted for proteolysis by other extracellular proteases. The Tat-dependently secreted protein YwbN is targeted for proteolysis by wall-bound WprA and various extracellular proteases. Blue arrows mark the secretion of WprA and YwbN.

In conclusion, the data described in this PhD thesis highlight important roles of the extracytoplasmic proteases of *B. subtilis* in (1) the degradation of native extracytoplasmic proteins and (2) the folding and quality control of these proteins. These roles impact on integral membrane proteins, lipoproteins and secreted proteins. Moreover, not only the proteins that are exported in an unfolded state via the Sec pathway are targeted by the extracytoplasmic proteases of *Bacillus*, but this is also true for at least one Tat-dependently secreted protein, namely YwbN. Notably, the biological functions of many extracytoplasmic proteins that were identified as targets of extracytoplasmic proteases of *B. subtilis* have not yet been identified. The identification of these functions, together with the generation of specific antibodies, would pave the way for more detailed studies on the importance and regulatory roles of proteases in the 'life and death' of the extracytoplasmic proteins of *B. subtilis*. The results of these future studies could then be applied for the construction of super-secreting synthetic *Bacillus* cells. Importantly, a better understanding of the biological roles of proteolytic activities at the Gram-positive bacterial cell surface can probably also provide important leads for the development of novel anti-infectives that are dearly needed in the continuous fight against major bacterial pathogens. It is thus foreseen that proteolysis of extracytoplasmic bacterial proteins will remain an important research topic in the years to come.

Samenvatting, algemene discussie en perspectief

Bacillus subtilis is een Gram-positieve staafvormige bacterie, die in de bovenste lagen van de grond en op de wortels van planten leeft. Deze bacterie wordt voortdurend uitgedaagd door veranderende omstandigheden in zijn leefomgeving en hij beschikt daarom over diverse mechanismen om hier effectief op te reageren. *B. subtilis* produceert bijvoorbeeld antibiotica, zoals sublancin 168, om met andere microorganismen te concurreren voor nutriënten, die in beperkte hoeveelheden aanwezig kunnen zijn [1, 2]. Ook kan hij zijn flagellen benutten om competitie met andere microorganismen te vermijden door te migreren naar meer aantrekkelijke leefmilieus [3, 4]. Door opname van K^+ en verhoogde synthese van proline en glycine betaine kan *B. subtilis* een hoge osmolariteit in zijn leefmilieu overleven [5-9] en dit illustreert heel mooi hoe deze bacterie fysiologische aanpassingen benut om zich te beschermen tegen potentieel gevaarlijke situaties. De ontwikkeling van competentie voor opname van vreemd genetisch material is een meer complexe aanpassingsstrategie, die de robuustheid van een *Bacillus* populatie kan verhogen door vergroting van de genetische diversiteit [10]. Als dergelijke aanpassingen onvoldoende zijn, dan kan *B. subtilis* extreme omstandigheden overleven in de vorm van spores, totdat de condities in het leefmilieu weer gunstiger geworden zijn [11, 12].

De vroegtijdige beschikbaarheid van de complete genoomsequentie van *B. subtilis* heeft ervoor gezorgd, dat deze bacterie zich in recente jaren tot een ideaal model voor de bestudering van Gram-positieve bacteriën heeft ontwikkeld [13, 14]. Bovendien maakt het natuurlijke vermogen om grote hoeveelheden eiwit te secreteren *B. subtilis* tot een ideaal modelorganisme voor biotechnologisch onderzoek naar gesecreteerde eiwitten en de cellulaire eiwitsecretiemachinerie. Dergelijk onderzoek is extreem waardevol gebleken voor de biotechnologische toepassing van *B. subtilis*, in het bijzonder met betrekking tot de secretie van eiwitten met commerciële waarde [15-19]. Tenslotte

is *B. subtilis* volkomen ongevaarlijk voor mens, dier en plant, waardoor deze bacterie de status van een 'Generally Recognized As Safe' (GRAS) microorganisme heeft gekregen. Hierdoor is de brede toepassing van *B. subtilis* als cellulaire fabriek voor de productie van waardevolle gesecreteerde eiwitten en vitamines zeer aantrekkelijk.

Hoofdstuk 1 van dit proefschrift geeft een beknopt overzicht van het 'secretoom' van *B. subtilis*. Het secretoom van een organisme omvat per definitie de secretiemachinerie plus alle gesecreteerde eiwitten [20]. Hoofdstuk 1 gaat ook in op de mogelijkheden voor 'secretoom-engineering' in *B. subtilis*. Dit is nodig om aan de voortdurend toenemende behoefte aan enzymen en andere eiwitten van biomedische en industriële waarde te kunnen voldoen. Eerdere inspanningen op dit gebied waren met name gericht op de optimalisatie van zogenaamde signaalpeptides, die bepaalde eiwitten naar de machinerie voor eiwittranslocatie in de cytoplasmamembraan dirigeren. Ook is er onderzoek gedaan naar de mogelijkheden voor optimalisatie van de eiwittransportmachinerie, waaronder de Sec en Tat translocases, en andere componenten van de desbetreffende eiwitsecretieroutes. Dergelijke inspanningen waren bijvoorbeeld gericht op de optimalisatie van extracytoplasmatische catalysatoren voor eiwitvouwing, zoals de peptidyl-prolyl cis/trans isomerase PrsA of de thiol-disulfide oxidoreductase BdbD. De alzo verbeterde post-translocationele eiwitvouwing is een interessante benadering om de opbrengst van gesecreteerde eiwitten te verbeteren. Een alternatieve en meer pragmatische aanpak behelst de reductie van eiwitafbraak. Hierbij is de aandacht met name gericht op proteases in de cytoplasmamembraan, de celwand en het exoproteoom [21]. Deze omvatten de membraangebonden proteases HtrA en HtrB, de celwandprotease WprA, en de gesecreteerde proteases Mpr, AprE, Bpr, Epr, NprB, NprE en Vpr. Een aantal van deze proteases, zoals HtrA en WprA, hebben een dubbele localisatie en

geproceste vormen hiervan worden ook in het exoproteoom aangetroffen. De bekende functies van deze tien verschillende proteases zijn nogal divers, maar ze overlappen ook ten dele. Kort samengevat hebben de HtrA en HtrB proteases een belangrijke rol bij de eiwitkwaliteitscontrole. De WprA protease is betrokken bij eiwitkwaliteitscontrole en afbraak, en de gesecreteerde proteases zijn van belang voor het vergaren van nutriënten en de processing van andere gesecreteerde eiwitten. Deze functies zijn samengevat in Hoofdstuk 1 ter introductie van het onderzoek, dat in dit proefschrift beschreven is.

De gesecreteerde proteases van *B. subtilis* worden overwegend in de stationaire groeifase geproduceerd, bijvoorbeeld als bepaalde voedingsstoffen in beperkende hoeveelheden beschikbaar zijn. In de natuurlijke habitats van *B. subtilis* zijn de gesecreteerde extracellulaire proteases AprE, Bpr, Epr, Mpr, NprB, NprE, VprA en WprA met name van belang voor de afbraak van dood organisch materiaal tot kleinere moleculen zoals peptides en aminozuren, die dan als voedingsstoffen voor groei kunnen dienen [22-32]. Mogelijk breken deze proteases ook toxische eiwitten en peptides af ter bescherming van de *Bacillus* cel. Rollen van de extracellulaire proteases bij de processing van andere geëxporteerde eiwitten, zoals het abundante celwandeiwit WapA, en autolysines zoals LytE en LytF zijn eveneens gerapporteerd [33, 34]. Tenslotte is ook bekend, dat extracellulaire proteases betrokken zijn bij de zogenaamde 'swarming' motiliteit, de processing van lantibiotica and de processing van het Phr pentapeptide proCSF [35-37].

De membraangebonden proteases HtrA en HtrB behoren tot de HtrA familie van de serine proteases, maar ze hebben daarnaast ook een chaperone-achtige activiteit [38]. De expressie van de *htrA* en *htrB* genen staat onder controle van het CssRS twee-componenten systeem, dat er voor zorgt dat de transcriptie van deze genen is verhoogd onder 'secretiestress' condities. Van *B. subtilis* HtrA is bekend, dat het zowel protease- als chaperone functies heeft en dit is

waarschijnlijk ook het geval voor HtrB [39, 40]. Op grond van proteomics en moleculair-biologische analyses is gepostuleerd dat YqxI, een *B. subtilis* eiwit met onbekende functie, een potentieel substraat is voor de chaperone-activiteit van HtrA [41]. In pathogene Gram-positive bacteriën, zoals *Staphylococcus aureus*, is aangetoond dat HtrA van belang is voor de pathogenese. Dit suggereert dat HtrA een sleutelrol vervult in de kwaliteitscontrole van belangrijke fitness- en virulentiefactoren [42].

Extracellulaire proteases zijn vaak verantwoordelijk voor de afbraak van gesecreteerde heterologe eiwitten, hetgeen kan leiden tot een aanzienlijk productverlies. Het gebruik van meervoudig protease-deficiënte stammen, zoals de WB600, WB700 en WB800 stammen, kan derhalve erg nuttig zijn om de opbrengest van dergelijke kwetsbare eiwitten te verhogen [43-45]. De overproductie van HtrA en HtrB onder secretiestress condities leidt eveneens tot de afbraak van gesecreteerde eiwitten. Zelfs overgeproduceerde membraaneiwitten kunnen afgebroken worden door deze twee membraan-gebonden proteases. Op grond van de beschikbare data kan geconcludeerd worden, dat de extracytoplasmatische proteases vooral eiwitten afbreken, die langzaam vouwen of die knipplaatsen voor proteases op hun oppervlak exponeren. Hierbij dient opgemerkt te worden, dat langzame vouwing en het exponeren van knipplaatsen vaker voorkomt bij heterologe eiwitten. Om `deze reden is afbraak door extracytoplasmatische proteases een belangrijke bottleneck in de productie van heterologe eiwitten [39, 46, 47].

Het onderzoek beschreven in dit proefschrift was gericht op de rollen van de acht extracellulaire proteases AprE, Bpr, Epr, Mpr, NprB, NprE, VprA en WprA, en de twee membraan-gebonden proteases HtrA en HtrB in de afbraak van geëxporteerde eiwitten van *B. subtilis*. Dit onderzoek was relevant omdat, tot op heden, erg weinig bekend was over de stabiliteit en het uiteindelijke lot van geëxporteerde *Bacillus* eiwitten. Het eerste experimentele

hoofdstuk van dit proefschrift beschrijft daarom de analyse van de exoproteomen van meervoudig protease-deficiënte mutante stammen (Hoofdstuk 2). Vervolgstudies beschreven in de twee andere experimentele Hoofdstukken 3 en 4 waren gericht op de rollen van extracytoplasmatische proteases bij de afbraak van afzonderlijke *B. subtilis* eiwitten. Hiertoe werd een compleet nieuwe set van stammen gebruikt met markervrije gen-deleties voor de tien voornoemde extracytoplasmische proteases. Deze stammen met de namen *B. subtilis* BRB02-14 werden beschikbaar gesteld door Dr. Rocky Cranenburgh en Prof. Colin Harwood.

Een eerste proteomics-analyse, gebaseerd op twee-dimensionale (2D) gelelectroforese (PAGE) van celwand-eiwitten van protease-deficiënte *B. subtilis* stammen, had belangrijke aanwijzingen opgeleverd ten aanzien van de rol van extracellulaire proteases, met name Epr, in de afbraak van een aantal celwandeiwitten van *B. subtilis* [34]. De totale omvang van de afbraak van celenvelop- en extracellulaire eiwitten bleef echter onopgehelderd. De oorzaak hiervan was dat 2D-PAGE-gebaseerde proteomics benaderingen niet gebruikt kunnen worden voor de analyse van eiwitten met extreem hoge of lage pI's, hydrofobe eiwitten, en kleine eiwit-isoformen, die ontstaan zijn door post-translationele processing of modificatie. Sommige van deze beperkingen kunnen vermeden worden door een combinatie van eiwitscheiding via één-dimensionale (1D) PAGE met 'liquid chromatography' (LC) en massaspectrometrie (MS) in de zogenaamde GeLC-MS benadering [48].

Hoofdstuk 2 beschrijft de toepassing van GeLC-MS voor de gedetailleerde analyse van de exoproteomen van stam BRB08, die de acht extracytoplasmatische proteases AprE, Bpr, Epr, Mpr NprB, NprE, Vpr en WprA mist, en stam BRB14, die deze acht extracytoplasmatische proteases *plus* de twee membraan-gebonden proteases HtrA en HtrB mist. Een gedetailleerde vergelijking van de exoprotemen van deze twee protease-

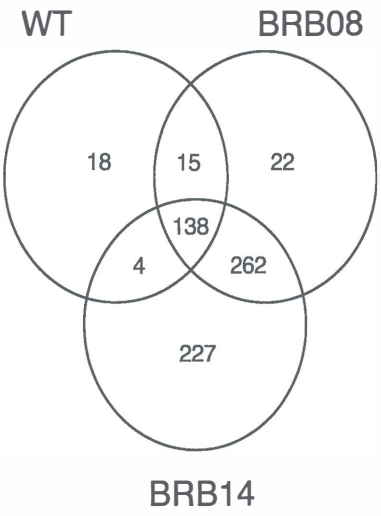
deficiënte strammen en de 'ouderlijke' stam 168 resulteerde in de identificatie van veel potentiële substraten van de extracellulaire en membraan-gebonden proteases van *B. subtilis*. Deze afgebroken eiwitten omvatten gesecreteerde eiwitten, lipoproteines en membraaneiwitten. De desbetreffende resultaten zijn samengevat in Figuur 1 en ze kunnen als volgt geïnterpreteerd worden:

- 138 geïdentificeerde eiwitten in het exoproteoom van *B. subtilis* 168 werden niet detecteerbaar beïnvloed door de tien extracytoplasmische proteases, die afwezig zijn in stam BRB14. Hierbij dient echter opgemerkt te worden, dat de resultaten van de gebruikte GeLC-MS benadering eerder kwalitatief dan kwantitatief zijn. Het is derhalve niet uit te sluiten, dat verschillende extracytoplasmatische proteases de absolute hoeveelheden van deze 138 eiwitten kunnen beïnvloeden. Dit blijkt inderdaad het geval te zijn voor een aantal lipoproteines, waartegen specifieke antilichamen beschikbaar waren voor Western blotting analyses (zie Hoofdstukken 2 en 4).
- 18 geïdentificeerde eiwitten waren slechts detecteerbaar in het exoproteoom van stam 168. Dit betekent dat hun aanwezigheid in het exoproteoom afhankelijk is van de activiteit van één of meerdere proteases, die afwezig zijn in de stammen BRB08 en 14. Het is voorstelbaar dat de WprA protease hierbij betrokken is, omdat deze een rol heeft in de kwaliteitscontrole van extracytoplasmatische eiwitten.
- 15 geïdentificeerde eiwitten waren slechts detecteerbaar in het exoproteoom van de stammen 168 and BRB08. Dit suggereert dat deze eiwitten HtrA en/of HtrB nodig hebben voor vouwing in een stabiele conformatie en/of voor hun uitscheiding in het groeimedium. Aangezien negen van deze eiwitten lipoproteines zijn is het aannemelijk dat HtrA en HtrB betrokken zijn bij het fenomeen 'lipoproteine shaving', dat in veel Gram-positieve bacteriën wordt

waargenomen (Figuur 2). Lipoproteine shaving is echter niet volledig geblokkeerd in stam BRB14, en dit duidt er op dat bepaalde membraanproteases, die niet in de huidige studies onderzocht werden, bij dit proces betrokken zijn.

- 22 geïdentificeerde eiwitten waren slechts detecteerbaar in het exoproteoom van stam BRB08. Dit suggereert that deze eiwitten substraten zijn van tenminste één van de acht proteases, die ontbreken in stam BRB08 en dat ze HtrA en/of HtrB voor hun vouwing in een stabiele conformatie nodig hebben.
- 262 geïdentificeerde eiwitten waren slechts detecteerbaar in het exoproteoom van de stammen BRB08 en 14. Dit zou kunnen betekenen, dat deze eiwitten substraten zijn van één of meerdere proteases, die afwezig zijn in de stam BRB08. De stammen BRB08 en 14 hebben echter last van verhoogde cel-lysis, zoals gebleken is uit de aanzienlijk verhoogde extracellulaire niveaus van het cytoplasmatische markereiwit TrxA. Het lijkt daarom eerder aannemelijk dat met name de 219 cytoplasmatische eiwitten in deze categorie gedetecteerd worden op grond van de verhoogde cel-lysis dan gereduceerde proteolyse, of een combinatie van deze twee effecten.
- 227 geïdentificeerde eiwitten waren slechts detecteerbaar in het exoproteoom van de stam BRB14. Het lijkt hoogst onwaarschijnlijk, dat al deze eiwitten substraten van HtrA en/of HtrB zijn. In plaats daarvan lijkt het meer aanemelijk, dat de aanzienlijk verhoogde lysis van stam BRB14 (vergeleken met stammen 168 en BRB08) leidt tot een dramatische ophoping van cytoplasmatische eiwitten in het groeimedium. Het lijkt daarom redelijker om alleen de typische membraaneiwitten (11), lipoproteines (2) en gesecreteerde eiwitten (1) in deze categorie als potentiële substraten van HtrA en/of HtrB te

beschouwen. Deze membraaneiwitten en lipoproteines kunnen echter ook door cel-lysis in het medium terecht komen, zoals bijvoorbeeld het geval lijkt te zijn voor het membraaneiwit BdbD (Figuur 2).



Figuur 1. Vergelijking van eiwitten in de exoproteomen van de meervoudig protease-deficiënte *B. subtilis* stammen BRB08 en 14, en hun ouderstam 168. Eiwitten in exoproteoom-monsters van de *B. subtilis* protease mutanten BRB08 (08) en BRB14 (14) of hun ouderstam 168 (wt) werden geïdentificeerd via GeLC-MS analyses (Hoofdstuk 2). Het Venn-diagram laat de aantallen eiwitten zien, die uniek zijn voor de geanalyseerde stammen, of die aangetroffen worden in twee of drie stammen.

4 geïdentificeerde eiwitten waren slechts detecteerbaar in de exoproteomen van de stammen 168 and BRB14. Volgens de hierboven gevolgde redenering voor interpretatie van de proteoomdata zouden deze vier eiwitten een protease nodig hebben voor stabiele vouwing, die afwezig is van stam BRB08 en aanwezig is in stam BRB14. Dit lijkt nogal

onwaarschijnlijk en het is veel aannemelijker dat deze vier eiwitten representatief zijn voor de mate van biologische variatie in de onderhavige proteoom-analyses.

Het is van belang om op te merken, dat het niet-detecteren van een eiwit in een proteoom-analyse niet noodzakelijkerwijs betekent, dat dit eiwit niet aanwezig is in het geanalyseerde monster. Een dergelijk eiwit kan ook eenvoudigweg ontsnapt zijn aan detectie door technische redenen, of door een bepaalde mate van 'ruis' tengevolge van biologische fluctuaties. Dit is waarschijnlijk ook het geval voor de vier eiwitten die uitsluitend in de media van de stammen 168 en BRB14 werden aangetroffen. Geëxtrapoleerd naar de volledige dataset zou dit suggereren, dat slechts een beperkt aantal 'afwezige eiwitten' in de verschillende monsters afwezig is tengevolge van technische beperkingen of biologische ruis. Dit is een belangrijke conclusie, die het vertrouwen in onze interpretatie van de rollen van extracytoplasmatische proteases in de exoproteoom-biogenese versterkt. In deze context, is het ook relevant om op te merken, dat de impact van meervoudige protease mutaties op de expressie van bepaalde genen nog niet is onderzocht. Het is daarom niet helemaal uit te sluiten, dat sommige waargenomen verschillen in de exoproteoom-samenstelling terug te voeren zijn op gen-regulatoire responsen. Dit is een aspect van het huidige promotieonderzoek, dat meer aandacht verdient in toekomstige studies. Een ander aspect betreft de beste manier om monsters the bereiden voor proteomics en Western blotting analyses. In Hoofdstuk 2 van dit proefschrift, zijn gelijke hoeveelheden eiwit op de gel geladen. Hoewel het gebruik van gelijke hoeveelheden eiwit van voordeel is voor gel-vrije proteomics analyses, heeft deze benadering nadelen voor gel-gebaseerde proteomics analyses, met name als verschillende monsters grote verschillen

laten zien, zoals het geval was voor de monsters van *B. subtilis* stam 168 en de meervoudige protease-mutant. Achteraf bezien was het laden van samples met een correctie voor de optische dichtheid van de verschillende cultures, zoals in Hoofdstukken 3 en 4, een betere optie geweest, met name voor de Western blotting analyses.

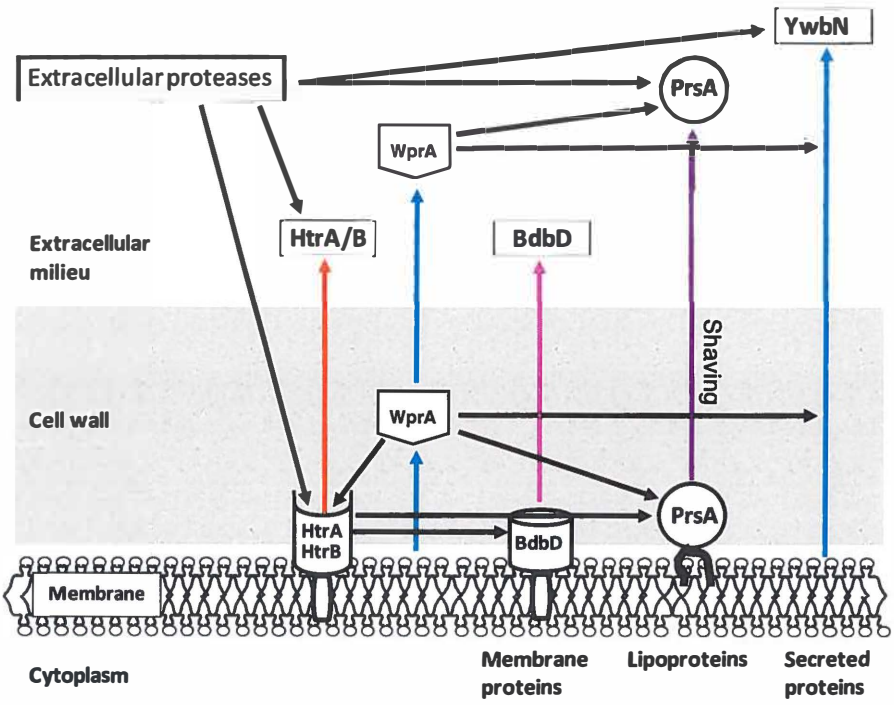
Op grond van de hoge gevoeligheid van stam BRB14 voor cel-lysis, veronderstellen we dat de afwezigheid van zowel HtrA als HtrB kan leiden tot verminderde afbraak van bepaalde membraaneiwitten. Dit zou kunnen leiden tot destabilisatie en uiteindelijke disruptie van de membraan, hetgeen vervolgens zou leiden tot het vrijkomen van cytoplasmatische eiwitten in het groeimedium. Alles tesamen geven de exoproteoom-analyses van *B. subtilis* stammen, die meerdere extracytoplasmische proteases missen, eerste waardevolle inzichten ten aanzien van de vraag welke eiwitten in belangrijke mate afgebroken worden en de resultaten zijn ook indicatief voor mogelijke regulatoire- en kwaliteitscontrole mechanismen, die de samenstelling van het extracellulaire proteoom van *B. subtilis* bepalen. Om de conclusies van de exoproteoom-analyses met meervoudige protease mutanten van *B. subtilis* te valideren werden de rollen van extracytoplasmatische proteases in meer detail onderzocht in de experimenten beschreven in Hoofdstukken 3 en 4 van dit proefschrift. **Hoofdstuk 3** documenteert studies naar de rol van extracytoplasmatische proteases in de afbraak van het YwbN eiwith van *B. subtilis*. Samen met de phosphodiesterase PhoD, is de Dyp-type peroxidase YwbN op dit moment het best-bestudeerde eiwit, dat via de *Bacillus* Tat route gesecreteerd wordt. Het YwbN eiwit wordt specifiek gesecreteerd via de TatAyCy translocase [49, 50]. Aangezien de Tat-route een voorkeur heeft voor de translocatie van volledig gevouwen eiwitten, zou men kunnen verwachten, dat extracytoplasmatische proteases Tat-afhankelijk gesecreteerde eiwitten, zoals YwbN, weinig kwaad kunnen doen. Dit idee is gebaseerd op het gegeven, dat

volledig gevouwen eiwitten in het algemeen veel minder gevoelig zijn voor proteolyse dan ongevouwen eiwitten. Verondersteld werd daarom, dat de secretie van eiwitten in een gevouwen staat via de Tat-route deze eiwitten veel minder gevoelig zou maken voor afbraak door de extracytoplasmatische proteases van *B. subtilis* [51]. Dit idee werd getest met behulp van de meervoudig protease-deficiënte stammen BRB02-BRB14. Geheel onverwacht werd een bijna lineaire toename in de niveaus van extracellulair YwbN waargenomen. Dit laat zien, dat dit Tat-afhankelijk gesecreteerde eiwit onderhavig is aan afbraak door meerdere extracellulaire proteases (Figuur 2). Inderdaad, kon via complementatie-analyses aangetoond worden, dat de AprE, Bpr, NprE en Vpr proteases betrokken zijn bij de afbraak van YwbN. Voorts werd in de cellulaire fracties van protease-deficiënte stammen zonder WprA een specifiek afbraakpatroon van YwbN waargenomen, hetgeen suggereert dat ook WprA betrokken is bij de afbraak van YwbN (Figuur 2). Deze bevindingen laten zien dat de extracellulaire niveaus van YwbN bepaald worden door proteolyse, hetgeen de aanwezigheid van een kwaliteitscontrolefunctie suggereert. Dit idee wordt ondersteund door recente data van Monteferrante *et al.*, die laten zien dat WprA een directe interactie kan aangaan met de TatAyCy translocase en een rol speelt bij de biogenese van YwbN (persoonlijke mededeling van Carmine Monteferrante). Tesamen suggereren de resultaten beschreven in Hoofdstuk 3, dat volledig gevouwen eiwitten, die via de Tat-route gesecreteerd worden, niet meer protease-resistent zijn dan eiwitten die in een ontvouwen staat via de Sec-route gesecreteerd worden. Meerdere verklaringen zijn mogelijk voor de waargenomen afbraak van YwbN. Dit eiwit zou bijvoorbeeld toch in een gedeeltelijk ontvouwen staat gesecreteerd kunnen worden. Ook is het denkbaar, dat knipplaatsen voor proteases op het oppervlak van gesecreteerd YwbN geëxponeerd zijn, of dat er een gereguleerde afbraak van YwbN plaatsvindt, al naar gelang er behoefte is aan de enzymatische activiteit van dit eiwit.

In **Hoofdstuk 4** worden de effecten van extracellulaire proteases op de extracytoplasmatische catalysatoren voor eiwitvouwing PrsA en BdbD, als ook de kwaliteitscontrolefactoren HtrA en HtrB beschreven. Het lipoproteine PrsA is een belangrijke vouwingscatalysator voor meerdere penicilline-bindende eiwittten (PBP2a, PBP2b, PBP3, and PBP4) [52]. Verschillende studies hebben laten zien, dat overproductie van PrsA leidt tot een aanzienlijk verbeterde secretie van verschillende α-amylases, het protective antigen van *Bacillus anthracis* en interferon-β (h-IFN β) [17, 53, 54]. De analyse van PrsA in cellen van meervoudig protease-deficiënte stammen liet zien, dat de afbraak van cel-geassocieerd PrsA sterk gereduceerd was in stammen die WprA missen (Figuur 2). Verhoogde hoeveelheden PrsA werden ook gedetecteerd in de media van de respectievelijke *wprA* mutante strammen, hetgeen impliceert dat PrsA in het groeimedium normaalgesproken afgebroken wordt door één of meerdere extracellulaire proteases (Figuur 2). Dit suggereert dat de toename in de secretie van bepaalde eiwitten in meervoudig protease-deficiënte *Bacillus* stammen, die geen WprA produceren, mogelijk het gevolg is van verhoogde PrsA niveaus en gereduceerde proteolyse. Hierbij dient opgemerkt te worden, dat deletie van alleen het *wprA* gen niet resulteerde in verhoogde cellulaire of extracellulaire PrsA niveaus. Dit betekent dat positieve effecten van de deletie van *wprA* aanzienlijk versterkt worden door de afwezigheid van andere extracytoplasmatische proteases. In tegenstelling tot PrsA waren de cellulaire niveaus van de thiol-oxidase BdbD, die nodig is voor disulfidebrug-vorming in extracytoplasmatische eiwitten van *B. subtilis* [55, 56], niet beïnvloed door meervoudige protease mutaties. Desalnietteming bevatten de media van de stammen BRB11 en BRB14 verhoogde BdbD niveaus. Aangezien deze twee stammen erg goevoelig zijn voor lysis suggereert deze waarneming, dat cel-lysis een belangrijke parameter is voor de release van BdbD van de cellen. Aan de andere kant is het niet volledig uit te sluiten, dat BdbD-afbraak verminderd

is door de afwezigheid van WprA en HtrA (Figuur 2), omdat de niveaus van extracellulair TrxA ook verhoogd waren bij de BRB12 en 13 stammen, terwijl deze stammen minder extracellulair BdbD leken te hebben.

Sterk verhoogde niveaus van HtrA of HtrB werden waargenomen in de cellulaire en groeimedium fracties van stammen, die *htrA* of *htrB* missen. Dit is waarschijnlijk gerelateerd aan de CssRS-afhankelijke kruis-regulatie van *htrA* en *htrB*. Vergelijkbaar met wat waargenomen was voor PrsA, waren de niveaus van HtrA en HtrB aanzienlijk verhoogd in cellen van meervoudig protease-deficiënte strammen zonder WprA. Dit suggereert, dat WprA betrokken is bij de afbraak van HtrA en HtrB (Figuur 2). Consistent met eerder gepubliceerde waarnemingen, werd een verhoogd HtrA niveau waargenomen in de groeimedia van strammen BRB02-08. Dit suggereert dat met name AprE and NprB verantwoordelijk zijn voor de extracellulaire afbraak van HtrA. Een bijna lineaire toename in de extracellulaire accumulatie van HtrB werd waargenomen voor de protease mutanten BRB02-BRB08. Dit impliceert dat HtrB gevoeliger is voor proteolyse dan HtrA en dat meerdere extracellulaire proteases betroken zijn HtrB afbraak. Het lijkt er dus op dat PrsA, HtrA en HtrB na hun release van de celmembraan door WprA afgebroken worden door één of meer extracellulaire proteases van *B. subtilis*. Deze waarnemingen laten zien, dat de celwand-gebonden en gesecreteerde protease WprA een nog grotere invloed heeft op de integriteit en kwaliteit van geëxporteerde eiwitten dan voorheen gedacht [57-60]. De verhoogde cellulaire niveaus van HtrA en HtrB in meervoudige protease-deficiënte cellen zonder WprA zullen wellicht bijdragen aan een verbeterde eiwitkwaliteitscontrole aan het celmembraanoppervlak, alwaar eiwitten uit het Sec-kanaal opduiken en snel moeten vouwen om niet afgebroken te worden.



Figuur 2. Schematische weergave van de extracytoplasmatische proteases en hun potentiële targets in *B. subtilis*. *B. subtilis* produceert tenminste acht extracellulaire proteases genaamd AprE, Bpr, Epr, Mpr, NprB, NprE, Vpr en WprA. De WprA protease is ook actief aan het membraanoppervlak en in de celwand. *B. subtilis* bezit daarnaast tenminste twee membraan-geassocieerde proteases genaamd HtrA en HtrB. De extracellulaire en membraan-gebonden proteases worden collectief extracytoplasmatische proteases genoemd. Potentiële targets van de extracytoplasmatische proteases werden geïdentificeerd via proteomics en Western blotting analyses (gemarkeerd met zwarte pijlen). HtrA en HtrB worden zelf afgebroken door de extracellulaire proteases en WprA, hetgeen leidt tot de release van deze eiwittten van de membraan (oranje pijl). HtrA of HtrB zijn betrokken bij de release en afbraak van het membraaneiwit BdbD (roze pijl). WprA en HtrA of HtrB zijn

betrokken bij de 'shaving' van het lipoproteine PrsA (paarse pijl). PrsA, dat in het groeimedium belandt wordt afgebroken door andere extracellulaire proteases. Het Tat-afhankelijk gesecreteerde eiwit YwbN wordt afgebroken door het celwand-gebonden WprA protease en verschillende andere extracellulaire proteases. Blauwe pijlen markeren de secretie van WprA en YwbN.

Samenvattend kan uit de resultaten van dit promotieonderzoek geconcludeerd worden, dat de extracytoplasmatische proteases van *B. subtilis* belangrijke rollen hebben in (1) de afbraak van natieve extracytoplasmatische eiwitten en (2) de vouwing en kwaliteitscontrole van deze eiwitten. Deze rollen zijn van invloed op integrale membraaneiwitten, lipoproteines en gesecreteerde eiwitten. Bovendien worden niet alleen eiwitten, die in een ontvouwen staat via het Sec-kanaal geëxporteerd worden door de extracytoplasmatische proteases van *Bacillus* afgebroken, maar ook tenminste één Tat-afhankelijk gesecreteerd eiwit, namelijk YwbN. Opgemerkt dient te worden, dat de biologische functies van veel extracytoplasmatische eiwitten, die als mogelijk substraat van extracytoplasmatische proteases van *B. subtilis* geïdentificeerd werden, nog niet bekend zijn. De identificatie van deze functies, gecombineerd met het opwekken van specifieke antilichamen tegen deze eiwitten, zal het pad effenen voor meer gedetailleerd onderzoek naar het belang en de regulatoire rol van proteases bij 'leven en dood' van de extracytoplasmatische eiwitten van *B. subtilis*. De resultaten van deze toekomstige studies zouden dan toegepast kunnen worden voor de constructie van super-secreterende synthetische *Bacillus* cellen. Een beter begrip van de biologische rol van proteolytische activiteiten op het celoppervlak van Gram-positieve bacteriën zal waarschijnlijk ook leiden tot belangrijke 'leads' voor de ontwikkeling van nieuwe antimicrobiële agentia, die hard nodig zijn in de continue strijd tegen

antibioticum-resistente bacteriële ziekteverwekkers. Het is aldus te voorzien dat proteolyse van extracytoplasmatische bacteriële eiwitten een belangrijk thema van onderzoek zal blijven in de komende jaren.

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